Altered renal function and the development of angiotensin II-dependent hypertension

By Md. Ali Ashek

Ph.D. Thesis
Department of Molecular Physiology
The University of Edinburgh
2011

I declare that all work presented in this thesis is my own, except where stated otherwise, and that it has been entirely compiled by myself.

Md. Ali Ashek

Acknowledgements

First of all I wish to thank my supervisor Professor John Mullins for his help, encouragement and support throughout the period of my study. I would also like to thank my other supervisor, Dr Matthew Bailey for his constant guidance from the beginning to the end of my project as well as for his enormous help to prepare this thesis.

I am truly grateful to Dr Peter Flatman for passing on his western blot wisdom. I would also like to express my gratitude to Dr Chris Kenyon for his invaluable help in tail cuff blood pressure measurement. I must thank Dr Gillian Gray and Dr Matthew Sharp for their constructive advice during my committee meetings.

I am grateful to Gillian Brooker for her help in the renal clearance study. I would also like to thank all the BRR/BRF staff for their help with my animals and experiments. Special thanks to Louise Evans for correcting my grammatical mistakes during the preparation of this thesis.

I am very grateful to ORSAS and the Post Graduate School for awarding me the scholarship, without which the work could not have been performed. I would also like to thank Dr. Rudolph Riemersma for his effort to bring me to the University of Edinburgh.

My unlimited thanks go to my beloved wife Mumu specially to let me come to the lab on weekends and work till late. I would also like to thank all my friends and family for their support and encouragement. Special thanks to Eilidh Craigie and Madina Kara for always being there and supporting me. Finally thanks to all the Molecular Physiology lab members for making my stay a very pleasant one.

Dedication

To my parents & wife

For their love, endless support and encouragement.

Abbreviation List

A

ACE -	Angiotensin Converting Enzyme
ACTH -	Adrenocorticotrophic Hormone
AGT -	Angiotensinogen
ANG I -	Angiotensin I
ANG II -	Angiotensin II
ANOVA -	Analysis of Variants
AT_1 -	Angiotensin Type I Receptor
AT_2 -	Angiotensin Type II Receptor

B

BP -	Blood Pressure
BSA -	Bovine Serum Albumin

C

cAMP-	Cyclic Adenosine Monophosphate
CCD -	Cortical Collecting Duct
CD -	Collecting Duct
C_{Inu} -	Clearance of Inulin
CNS -	Central Nervous System
CNT -	Connecting Tubule

D

DCT -	Distal Convoluted Tubule
DMSO -	Dimethyl Sulfoxide
DOCA -	Deoxycorticosterone Acetate
DTT-	1,4-dithiothreitol

E

ECF -	Extracellular Fluid
ECV -	Extracellular Volume
ELISA -	Enzyme-Linked Immunosorbent Assay
E_{Na} -	Sodium Excretion
ENaC -	Epithelial Sodium Channel

F

FE_{Na} - Fractional Excretion of Sodium

FE_K - Fractional Excretion of Potassium

FE_{Li} - Fractional Excretion of Lithium

FF - Filtration Fraction

FITC- Fluorescein isothiocyanate

G

GFR - Glomerular Filtration Rate

H

H_2O_2 - Hydrogen Peroxide

HRP- Horseradish Peroxidase

I

I3C- Indole 3 Carbinol

ICF - Intracellular Fluid

IMCD - Inner Medullary Collecting Duct

I.P. - Intraperitoneal

I.V. - Intravenous

K

Kb - Kilobases

M

MABP - Mean Arterial Blood Pressure

MD- Macula Densa

MR - Mineralocorticoid Receptor

N

Na-K-ATPase - Sodium Potassium Pump

NCC - Sodium Chloride Symporter

Nedd - Neuronal precursor cell Expressed Developmentally Down-regulated

NHE- Sodium Hydrogen Exchanger

NKCC - Sodium Potassium Chloride Symporter

NO - Nitric Oxide

P

PA - Primary Aldosteronism

PAH - p-Aminohippuric Acid

P_{Inu} - Plasma concentration of Inulin

P_{PAH} - Plasma concentration of p-Aminohippuric Acid

PMSF - Phenylmethylsulfonyl Fluoride monohydrate

P_{Na} - Plasma Sodium Concentration

ppm - parts per million

PRA - Plasma Renin Activity

PVDF - Polyvinylidene Difluoride

R

RAAS - Renin Angiotensin Aldosterone System

RBF - Renal Blood Flow

ROMK - Renal Outer Medullary Potassium (K) channel

RPF - Renal Plasma Flow

RVR - Renal Vascular Resistance

S

SBP - Systolic Blood Pressure

SGK- Serum- and Glucocorticoid-inducible protein Kinase

SHR - Spontaneously Hypertensive Rat

T

TAL- Thick Ascending Limb

TCA - Trichloroacetic Acid

U

U_{Inu} - Urinary Inulin concentration

U_{PAH} - Urinary p-Aminohippuric Acid concentration

V

VSMC - Vascular Smooth Muscle Cell

W

WNK - With no lysine (K)

Table of Contents

Abstract

Inappropriate modulation of the renin angiotensin system (RAS) can lead to derangements of blood pressure homeostasis in humans. Cyp1a1-mRen2.F transgenic rats were used to define the renal mechanisms underlying the development of angiotensin II-dependent hypertension. These transgenic rats were previously generated by introducing the mouse Ren2 gene into the rat genome under the control of a Cyp1a1 inducible promoter. The aim of the current investigation was to establish the contribution of renal function to the development of hypertension in the Cyp1a1-mRen2.F transgenic rat.

Expression of the mRen2 transgene was induced by daily gavage of indole 3 carbinol (I3C) at the dose of 100mg/kg. Blood pressure was measured in conscious rats after 1, 3 or 7 days of treatment. The control group received the vegetable oil carrier for 7 days. In addition blood pressure, renal haemodynamics and excretory function were measured under thiobutabarbital anaesthesia.

Transgene induction caused a progressive increase in blood pressure in a time dependent manner. Neither glomerular filtration rate nor renal blood flow was affected. This indicates proper function of renal autoregulation during the experimental time course. Tubular sodium reabsorption was significantly increased after the first day of transgene induction and this effect was sustained for the duration of treatment. A pharmacological approach was used to localize the increased reabsorption to a specific region of the nephron and was found to reflect increased activity of the thiazide-sensitive cotransporter (NCC). Chronic administration of

thiazide significantly blunted the hypertensive response to transgene induction. Similarly AT_1 receptor blockade attenuated the hypertensive phenotype and prevented the transgene-induced stimulation of NCC activity. In contrast, mineralocorticoid receptor blockade did not prevent the development of either hypertension or increased NCC activity.

The current study suggests that the development of angiotensin II-dependent hypertension is mediated by increased tubular sodium reabsorption. Increased activity of NCC is a key hypertensive mechanism in this model and results directly from the actions of angiotensin II at the AT_1 receptor; indirect aldosterone pathways do not play a major role.

Chapter 1

Introduction

1.1 Hypertension

Hypertension or high blood pressure is a condition in which the blood pressure is chronically elevated and remains greater than the accepted upper range of normal blood pressure. According to British Hypertension Society guidelines, blood pressure below 120mmHg systolic and 80 mmHg diastolic is considered optimal. Blood pressure between 120/80mmHg and 139/89mmHg is termed prehypertension and a blood pressure of 140/90mmHg or over is called hypertension (Williams *et al*., 2004). Hypertension can be subdivided into primary hypertension and secondary hypertension. Primary or essential hypertension is a condition in which the cause is undetermined or unknown. This accounts for 90-95% of all cases of high blood pressure. Secondary hypertension is high blood pressure that results from a underlying known and often curable cause such as kidney disease, adrenal disorders, thyroid disorders, oral contraceptives, alcohol, amongst others.

Hypertension is one of the main modifiable risk factor for cardiovascular disease, ischaemic heart disease and renal diseases (Whitworth *et al*., 2003). As such, it represents a leading cause of mortality and affects approximately 20% to 30% of the world's population. Furthermore, by 2025 it is predicted that 60% of the total population will be affected by hypertension (Kearney *et al*., 2005). The prevalence of hypertension in UK has been reported to be approximately 42% in the population aged from 35 to 64 (Wolf-Maier *et al*., 2003). Hypertension is estimated to cause ~4.5% of global disease burden and is equally widespread in both the developed and developing world (Lawes *et al*., 2008). Thus hypertension is an important global

public health challenge and the therapeutic management of high blood pressure represents a major economic burden in most countries.

Better management of hypertension can reduce the risk of stroke by 40% and the risk of myocardial infarction by 15% (Collins *et al*., 1990). Improved hypertension management reduces the prevalence of cardiovascular disease but in a significant proportion of individuals it is inadequately managed (Chobanian, 2009). With current poor hypertension control rates worldwide, more individuals are being referred for resistant or refractory hypertension. Thus a better understanding of the genesis of hypertension would ultimately aid in the development of targeted therapeutics for the treatment of hypertension.

1.1.1 Pathogenesis of hypertension

In simple terms, hypertension can be caused either by increased blood volume (volume expansion) or by a reduction in the capacitance of the cardiovascular system (Navar, 1997). The kidney plays a major role in hypertension induced by volume expansion. In this setting, the kidneys fail to respond adequately to increased intravascular volume or have an impaired excretory capacity causing sodium and fluid retention (de Wardener, 1990, Hamlyn *et al*., 1986). In either case, sustained volume expansion in-turn increases cardiac output, leading to an over perfusion of tissues. The autoregulatory resistance from over perfused tissues then leads to an increase in peripheral resistance. This contributes further to increase in total resistance and causes hypertension (Coleman & Guyton, 1969, Cowley, 1992). That impaired renal natriuretic ability is key for the development of hypertension is

supported by both clinical and experimental data (Woolfson & Wardener, 1996). In patients with essential hypertension, for example, renal sodium excretion is markedly attenuated in response to intravenous saline load. The sodium retention is associated with an impairment of pressure natriuresis (Manunta *et al*., 1999). Kidney cross-transplantation studies have provided strong evidence in support of the kidney's pivotal role in essential hypertension. Normalization in blood pressure was observed in hypertensive patients following bilateral nephrectomy and kidney transplants from normotensive donors (Curtis *et al*., 1983). The incidence of hypertension in transplant recipients depends on the familial incidence of hypertension in the donor's family (Guidi *et al*., 1996). In experimental animals, several kidney cross-transplantation studies have also showed that normotensive recipient rats develop hypertension following a renal graft from genetically hypertensive donor rats (Bianchi *et al*., 1975, Heller *et al*., 1993, Rettig *et al*., 1989). Thus the pathogenesis of hypertension is primarily controlled by the kidney.

Hypertension can also be caused by increased peripheral resistance or decreased cardiovascular capacitance. This can be caused by excess secretion of vasoconstrictors such as ANG II (Ashton, 2007; Mitchell *et al*., 1992). Again over-activation of sympathetic nerves also causes hypertension through this mechanism (Julius & Nesbitt, 1996). However, the Guyton Hypothesis (Guyton, 1991) would predict that increased blood pressure caused by either mechanism cannot be sustained if the pressure natriuresis response is normal: increased blood pressure would promote excretion of sodium and reduction in blood volume until arterial pressure returned completely to the original set point. However, some

vasoconstrictors can also cause renal sodium retention, leading to a profound hypertensinogenic stimulus. For example, abnormal activation of the RAAS causes an ANG II-dependent vasoconstriction, increasing total peripheral resistance. In addition, both ANG II and aldosterone promote sodium retention. The dampening of the pressure natriuresis response is synergistic to the vasoconstriction and produces a powerful hypertensinogenic stimulus (Mitchell *et al*., 1992).

Although it is well established that the kidney plays a key role in the pathogenesis of hypertension, there is evidence that hypertension can occur independently of the kidney. Vascular smooth muscle cell dysfunction may contribute to the pathogenesis of hypertension by impacting peripheral resistance without direct participation of the kidney (Tang *et al*., 2003). Several reports indicate that defects in ion channel function or dysregulation in the signaling pathway may cause chronic blood pressure elevation (Brenner *et al*., 2000, Zhu *et al*., 2002).

1.1.2 Genetic components for hypertension

There is substantial evidence that genetic factors contribute to blood pressure and hypertension. The normal distribution of blood pressure in the general population suggests a complex interaction between environmental and genetic factors. Twin and family aggregation studies also indicate the presence of a genetic component (Carretero & Oparil, 2000, Luft, 2001). In fact approximately 30% of blood pressure variation is attributed to genetic factors (Beevers *et al*., 2001). Population studies demonstrate greater similarity of blood pressure within families than between

families (Havlik *et al*., 1979, Longini *et al.*, 1984). In individuals who have one or two hypertensive parents, hypertension is twice as common as in the general population. Moreover twin studies suggest that blood pressure is more closely correlated in monozygotic than dizygotic twins (Carretero & Oparil, 2000, Luft 2001). This familial aggregation of blood pressure is not simply due to environmental factors. According to the Montreal Adoption Study, blood pressure is more closely correlated among biological siblings than adoptive siblings living in the same household (Mongeau *et al*., 1986).

Genetic studies have identified a number of gene mutations which have a large effect on blood pressure. These rare monogenic forms of hypertension and hypotension are all associated with major defects in renal salt handling (Lifton, 1996). More recently, it has been shown that minor variations in these genes are associated with blood pressure variability in the general population and thus may contribute to the genesis of essential hypertension (Lifton *et al*., 2001). There have been several genome wide association studies (GWAS) attempting to identify candidate genes for hypertension. The Wellcome Trust Case Control Consortium (WTCCC) conducted a GWAS for seven complex diseases of major public health burden, including hypertension (Wellcome Trust Case Control Consortium, 2007). However this study failed to identify any Single Nucleotide Polymorphism (SNP) associated with hypertension. Moreover there was no evidence for previously identified variants linking with hypertension. Two recent studies, Global Blood Pressure Genetics (Global BPgen) and Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) have produced more promising results. Global BPgen identified 8 loci associated

with blood pressure (Newton-Cheh *et al*., 2009). CHARGE identified multiple loci association with levels of systolic and diastolic blood pressure and hypertension (Levy *et al*., 2009). Joint analysis of results from the two consortia identified 11 genome-wide significant associations (Hastie *et al*., 2010; Newton-Cheh *et al*., 2009; Levy *et al*., 2009). Despite this, GWAS have failed to find strong associations between hypertension and specific genes, suggesting that there is no single locus responsible for substantial effects on blood pressure. This suggests that directed investigation of pathways such as the RAS, which are known to be involved in the regulation of blood pressure, may be more beneficial than the identification of multiple loci which impart very small effects on blood pressure in the population.

1.2 Renin angiotensin aldosterone system

The renin angiotensin aldosterone system (RAAS) plays an important role in blood pressure regulation and fluid homeostasis. It also plays a vital role in the pathophysiology of several diseases such as hypertension, cardiac hypertrophy, myocardial infarction and various progressive renal diseases. The evidence for this comes from genetic studies, identifying associations between angiotensinogen gene variants and hypertension in several European and Japanese population studies (Jeunemaitre *et al*., 1992, Hata *et al*., 1994). Evidence for a renin intronic dimorphism and essential hypertension has been reported in the United Arab Emirates population (Ahmad *et al*., 2005). The level of plasma and intracellular ACE is modulated by common insertion/deletion polymorphisms of the gene (Soubrier & Cambien, 1994, Cox *et al*., 2002). Marginal significance has been reported between

blood pressure and insertion/deletion polymorphisms from two large population-based studies and this linkage was restricted to males only (Fornage *et al*., 1998, O'Donnell *et al*., 1998). Furthermore the Framingham Heart Study found strong evidence for a quantitative trait locus on chromosome 17, close to the ACE gene (Levy *et al*., 2000). An association between angiotensin receptor variants and hypertension was reported in a study performed on a Finnish population (Kainulainen *et al*., 1999), but no association was observed in African Americans (Bonnardeaux *et al*., 1994, Gainer *et al*., 1997). Pharmacological evidence also supports a role of the RAS in essential hypertension: renin inhibitors, ACE inhibitors and AT_1 receptor antagonists are effective anti-hypertensive agents, even when overt signs of RAS activation are lacking (Ibrahim, 2006, Dzau *et al*., 2001, Pinto & Gradman, 2009).

In brief, the first step of the classical RAS is the cleavage of angiotensinogen, secreted from liver, by the enzyme renin, released from juxtaglomerular cells of the kidney, to produce angiotensin I (ANG I). This inactive intermediate is then further processed by a metalloprotease produced from the lung, angiotensin-converting enzyme (ACE), to produce the major effector of the RAS, angiotensin II (ANG II). Upon binding with its receptor (AT_1) ANG II directly exerts its physiological effect such as blood pressure regulation. ANG II also regulates blood pressure indirectly by inducing aldosterone synthesis from adrenal cortex. A schematic diagram of the enzymatic cascade of the renin-angiotensin-aldosterone system is illustrated in figure 1.1.

1.2.1 Angiotensinogen

Plasma angiotensinogen is the circulating protein substrate for renin in all species. The primary site for angiotensinogen synthesis is the liver, from where it is secreted constitutively (Li and Brasier, 1996). Angiotensinogen is also expressed, albeit at lower levels, in the central nervous system, kidney, heart

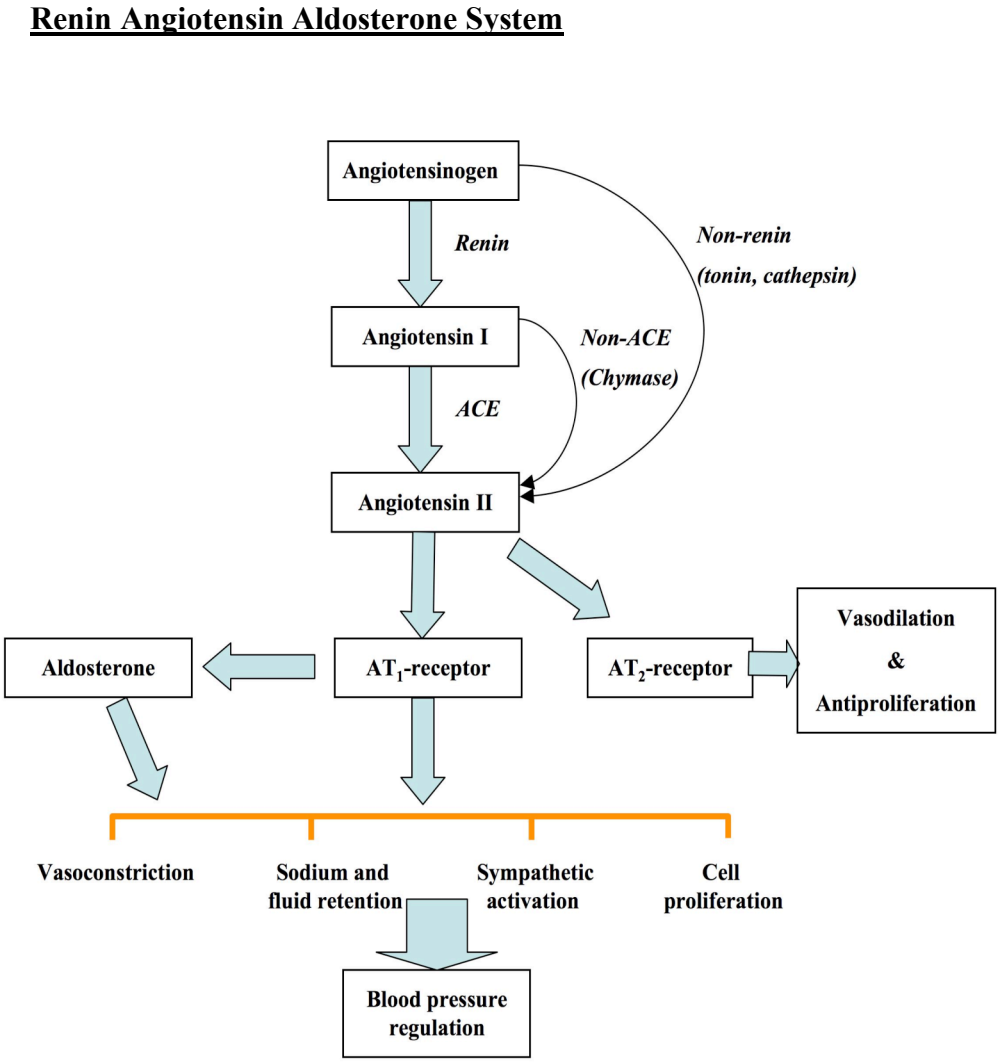


Figure 1.1 Enzymatic cascade of the renin-angiotensin aldosterone system and its role on blood pressure regulation

and adrenal gland (Griendling *et al*., 1993). No other function of angiotensinogen has been reported other than as a precursor of ANG II. Angiotensinogen synthesis is markedly elevated in response to stress such as tissue injury (Morgan *et al*., 1996). It is also stimulated by hormones such as glococorticoids, thyroid hormones and androgens (Morgan *et al*., 1996). ANG II stimulates the hepatic production of angiotensinogen by a positive feed back loop (Blair-West *et al*., 1974; Jaramillo *et al*., 1987, Nasjletti & Masson, 1973). Conversely renin inhibits angiotensinogen release (Herrmann & Dzau, 1983).

1.2.2 Renin

Renin is a very specific protease which catalyzes the cleavage of angiotensinogen to ANG I. Renin is secreted from specialized cells of the afferent arterioles called juxtaglomerular (JG) cells (Griendling, *et al*., 1993, Hackenthal *et al*., 1990). The JG cells synthesize an inactive precursor, called pre-prorenin, which is then processed into prorenin by the cleavage of a single amino acid from the C-terminus. Prorenin is also biologically inactive and further glycosylation and proteolytic cleavage leads to formation of renin (Dzau *et al*., 1988). Circulating renin is mostly derived from the JG cells but renin production has also been detected in several organs (Griendling, 1993).

Several mechanisms are involved in renin secretion from the JGA. Renin secretion is stimulated in response to decreased pressure or stretch; conversely increased pressure inhibits renin secretion (Wagner & Kurtz, 1998). Renin release is also regulated by the tubular fluid composition at the macula densa (MD) (Castrop *et al*., 2010). The macula densa is an area of closely packed specialized cells lining the wall of the

distal tubule at the point of return of the nephron to the afferent arteriole of its parent glomerulus. It has been shown that renin secretion is inhibited following renal arterial infusion of NaCl. Since MD cells do not have direct contact with the JGA, secondary messenger signalling is require to transmit the signal from the MD to the JGA. It has been shown that adenosine release from MD cells acts as a chemical signal to inhibit renin release from the JGA (Komlosi *et al*., 2004,). Furthermore renin release is also controlled by the sympathetic nervous system, several endocrine and paracrine hormones and extracellular Ca^{++} concentration (Castrop *et al*., 2010, Hackenthal *et al*., 1990).

There is ample evidence for the existence of tissue RAS in addition to circulating RAS. All the components of the RAS are available in several organ systems such as the brain, heart, adrenal gland and kidney (Bader *et al*., 2001; Morimoto & Sigmund, 2002). These local RAS also play a vital role in blood pressure regulation. It has been reported that antihypertensive effects of ACE inhibition are better correlated with tissue ACE inhibition rather than plasma ACE. Furthermore RAS inhibitors have also proved effective in treating hypertensive patients with normal or low levels of circulating RAS (Dzau *et al*., 2001). Intrarenal RAS has a regulatory role on blood pressure through the modulation of renal function (Navar *et al*., 1997) whereas brain RAS modulates blood pressure by facilitating neurotransmission and releasing vasopressin (Costa & Majewski, 1988; Steckelings *et al*., 1992).

A series of transgenic studies have been used to address the importance of the tissue or paracrine RAS. Double transgenic mice expressing kidney androgen-regulated hAGT and a systemically expressed hREN were reported to have increased blood

pressure with normal circulating ANG II levels following testosterone treatment (Ding *et al*., 1997). The increase in blood pressure was paralleled by a high concentration of urinary hAGT. Similarly when mice expressing hAGT under a brain-specific promoter were crossed with mice expressing hREN systemically, a 15mm Hg increase in blood pressure was reported (Morimoto *et al*., 2001). These observations clearly indicate the contribution of local RAS in blood pressure regulation.

The renin locus is an area of research interest. In humans, rats and some mouse strains, there is only one renin gene. Other mouse strains, however, have two renin genes at this locus, designated Ren1d and Ren2 (Mullins *et al*., 1982). This is thought to have arisen from a gene duplication event approximately 3 million years ago in the mouse lineage (Dickinson *et al*., 1984). Studies in two renin gene mice exemplify the role of renin in blood pressure control. Mice with two renin genes have much higher plasma renin concentrations compared to one renin gene carrying mice. Blood pressure is also greater in mice with two renin genes (Wang *et al*., 2002).

1.2.3 Angiotensin converting enzyme

Angiotensin converting enzyme (ACE) is a dipeptidyl carboxipeptidase, which cleaves a dipeptide from the carboxyl terminal of ANG I to form the octapeptide ANG II (Edros, 1975; Peach, 1977). ACE is predominantly synthesized in the pulmonary vasculature. ACE is also enzymatically active for a range of other small peptides such as inactivation of bradykinin. Thus, biologically, the actions of ACE potentiate vasoconstriction by producing ANG II and inactivating the vasodilator bradykinin. ACE has also been detected in the glomerular endothelial cells and

proximal tubule of the kidney (Paul *et al*., 2006). A homolog of ACE, termed ACE2, has been detected in the heart, kidney and testis (Donoghue *et al*., 2000). ACE2 also cleaves ANG I but it removes only a single peptide from the COOH-terminal to generate ANG-(1-9) whose function is unknown (Vickers *et al*., 2002). Subsequently ANG-(1-9) undergoes further cleavage by ACE to yield ANG-(1-7) which exerts a vasodilatory effect (Ferrario *et al*., 1997; Ren *et al*., 2002) upon binding with Mas receptors (Lemos *et al*., 2005). Moreover ACE2 also yields ANG-(1-7) by direct degradation of ANG II (Rice *et al*., 2004). Angiotensin II can also be generated from angiotensin I by a serine protease chymase (Paul *et al*., 2006). In addition, non-renin enzymes, including tonin and cathepsin are able to generate angiotensin II directly from angiotensinogen (Urata *et al*., 1994; Urata *et al*., 1996).

1.2.4 Angiotensin II

Angiotensin II (ANG II) is the primary active component of the RAS. ANG II has a very short biological half-life and its biological actions are mediated upon binding with its receptors. Although ANG II is the main effector of the RAS, some biologically active components may also be formed by further cleavage of ANG II by amonipeptidase (Ahmad & Ward, 1990) or by endopeptidase (Welches *et al*., 1991; Yamamoto *et al*., 1992). Fragmented peptides such as ANG III (2-8) and ANG IV (3-7) produced by aminopeptidase-mediated cleavage have the same action as ANG II although they are less potent (Goodfriend and Peach, 1975). ANG III is considered as the main effector of the RAS in the brain (Harding & Felix, 1987). On the other hand endopeptidase-mediated cleavage of ANG II produces ANG (1-7)

which is a vasodilator and causes natriuresis and diuresis (Handa *et al*., 1996; Vallon *et al*., 1998).

1.2.5 ANG II receptors

At the cellular level, action of ANG II is mediated mainly by two G-protein coupled receptors AT_1 and AT_2. The effects of ANG II to maintain blood pressure are mainly mediated by AT_1 receptors (Timmermans *et al*., 1993). In rodent two subtypes of AT_1 receptors termed AT_{1A} and AT_{1B} have been identified and they share substantial sequence homology. However AT_{1A} receptors are predominant and considered the closest homologue to the single human AT_1 receptor (Burson *et al*., 1994). AT_1 receptor activation stimulates phospholipase C, which in turn produces two secondary messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates the release of Ca^{++} from intracellular storage and DAG induces protein kinase C (PKC) activity, both of which cause vasoconstriction (Capponi, 1996; Griendling *et al*., 1996). In several tissues AT_1 receptor binding mediates inhibition of adenylate cyclase. This attenuates the production of the pro-vasodilation secondary messenger cAMP, thereby potentiating the direct vasoconstriction effects of ANG II (Apfeldorf & Rasmussen, 1988; Pobiner *et al*., 1985). The AT_1 receptor also activates phospholipase A_2 and D which leads to the production of prostaglandins which partially attenuates the vasoconstrictor effect of ANG II (Capponi, 1996; Griendling *et al*., 1996). Moreover, AT_1 receptor binding stimulates the influx of Ca^{++} into the cell by the opening of Ca^{++} channels. These mechanisms have been linked to aldosterone production and release (Apfeldorf & Rasmussen, 1988).

AT_1 receptors are expressed in a variety of organ systems such as the brain, adrenal gland, heart, vasculature and kidney. Actions in each of these systems serve to maintain blood pressure and fluid and electrolyte balance. In the vascular system, ANG II stimulation of the AT_1 receptor causes potent vasoconstriction resulting to an increase in blood pressure (Ito *et al*., 1995). In the adrenal cortex, AT_1 receptor activation mediates the secretion of aldosterone, which in turn causes the tubules of the kidneys to retain sodium and water (Masilamani *et al*., 1999). In the brain, pressor responses are observed upon intraventricular injection of ANG II, an effect mediated by AT_1 receptors (Davisson *et al*., 2000). In the kidney, AT_1 receptor activation by ANG II causes renal vasoconstriction and influences sodium and water reabsorption in several sections of the nephron. Moreover, AT_1 activation inhibits renin secretion by the JGA and thus exerts a negative feedback on the RAS: the so called "short-loop" feedback mechanism (Ichikawa & Brenner, 1980).

The AT_2 receptor is found in several organs such as the heart (Tsutsumi *et al*., 1998; Wharton *et al*., 1998), glomeruli, renal tubules and renal blood vessels (Ozono *et al*., 1997) and the adrenal medulla (Allen *et al*., 1999; Breault *et al*., 1996). The functional role of the AT_2 receptor is not fully characterized but several studies have described contributions to apoptosis, antiproliferation, differentiation and vasodilation (Csikos *et al*., 1998; Horiuchi, 1996). In the kidney, AT_2 receptors are involved in the production of cGMP, NO and prostaglandin and might, therefore, have a role in blood pressure regulation antagonistic to that of the AT_1 receptor-mediated pathways (Siragy & Carey 1996; Siragy & Carey 1997). The countervailing influence of AT_2 receptors is also apparent in terms of hypertensive organ damage. In the kidney, for example, high levels of angiotensin II cause

glomerular and tubulointerstitial injury which are mediated by AT_1 receptors. In contrast, over expression of the AT_2 receptor exerts a counter-regulatory mechanism to the adverse effects induced by AT_1 receptor in kidney disease. (Tejera e*t al.*, 2004; Vazquez *et al*., 2005)

1.3 Renal sodium handling

1.3.1 Role of renal haemodynamics

Renal blood flow (RBF) and glomerular filtration rate (GFR) are the two major determinants of renal haemodynamics. RBF is the volume of blood delivered to the kidneys per unit time. RPF is the rate at which plasma flows through the kidney. By estimating the rate of plasma flow through the kidneys, one can obtain an estimate of the rate of total blood flow through the kidneys. The normal rate of plasma flow through both kidneys of a 70 kg human is about 650 ml/min, and the normal rate of blood flow is about 1200 ml/min. Plasma makes up about 55% of the total blood volume and the plasma flow rate is about 55% of the total blood flow rate. Renal blood flow is not greatly influenced by systemic blood pressure due to autoregulation, (see section 1.3.1.2, below) and thus GFR, the volume of fluid that filters out of the plasma through the glomerular capillary walls into Bowman's capsules is influenced chiefly by factors that determine the balance of Starling's pressures across the capillary wall. These pressures are i) hydrostatic pressure, the net effect of which is to favour filtration; and ii) capillary oncotic pressure whereby the retention of proteins in the plasma opposes filtration.

1.3.1.1 Regulation of GFR

GFR is regulated by the sympathetic nervous system, hormones and autocoids. These factors control GFR by intrarenal changes in RBF: the relative constriction of the pre- (afferent) and post-(efferent) glomerular arterioles will determine glomerular hydrostatic pressure and thus the force favouring filtration. In addition, vasoactive agents can change the glomerular surface area available for filtration by inducing contraction or relaxation of the glomerular mesangial cells (Mitchell *et al*., 1992). Increased afferent arteriolar constriction reduces renal blood flow and hydrostatic pressure in the glomerular capillaries (P_{GC}), causing a reduction in GFR. Efferent arteriolar constriction generally increases glomerular hydrostatic pressure thus increases GFR. However a reduction in renal blood flow causes an increase in glomerular colloid osmotic pressure. As a consequence, during severe efferent arteriole constriction, the colloid osmotic pressure exceeds glomerular hydrostatic pressure and the net force for filtration actually drops which causes GFR to reduce. A reduction in glomerular capillary filtration coefficient reduces GFR.

1.3.1.2 Autoregulation of GFR and RBF

Increased arterial pressure tends to increase glomerular hydrostatic pressure, which increases GFR. However the intrinsic feedback mechanism of the kidney maintains RBF and GFR relatively constant. This feedback mechanism is termed autoregulation. Two mechanisms interact to provide highly efficient renal autoregulation: the tubuloglomerular feedback mechanism and the myogenic mechanism.

1.3.1.2.1 Tubuloglomerular feedback

In the tubuloglomerular feedback (TGF) mechanism, macula densa (MD) cells in the initial portion of the distal tubule sense the NaCl concentration of the tubular fluid and influence filtration at the glomerulus of origin. Any alterations that cause increased sodium reabsorption before the MD result in a decrease in the concentration of NaCl at the MD cells. This induces a signal from the MD cells that has two effects. It increases glomerular hydrostatic pressure by decreasing afferent arteriole resistance (Braam *et al*., 1993; Navar *et al*., 1996). This also causes vasoconstriction of the efferent arteriole by increasing renin secretion and consequently the formation of ANG II (Mitchell, 1992; Mitchell & Mullins, 1995). As a result of the synergistic effects of theses two events, GFR returns toward normal, which ultimately returns distal sodium delivery towards normal, so that sodium and water excretion can be maintained. An opposite series of action occurs when proximal tubular reabsorption is reduced. By this mechanism TGF allows precise control of the renal excretion of sodium and water. A general structure of the glomerulus along with the afferent and efferent arteriole, the macula densa cells and the JG cells is shown in figure 1.2.

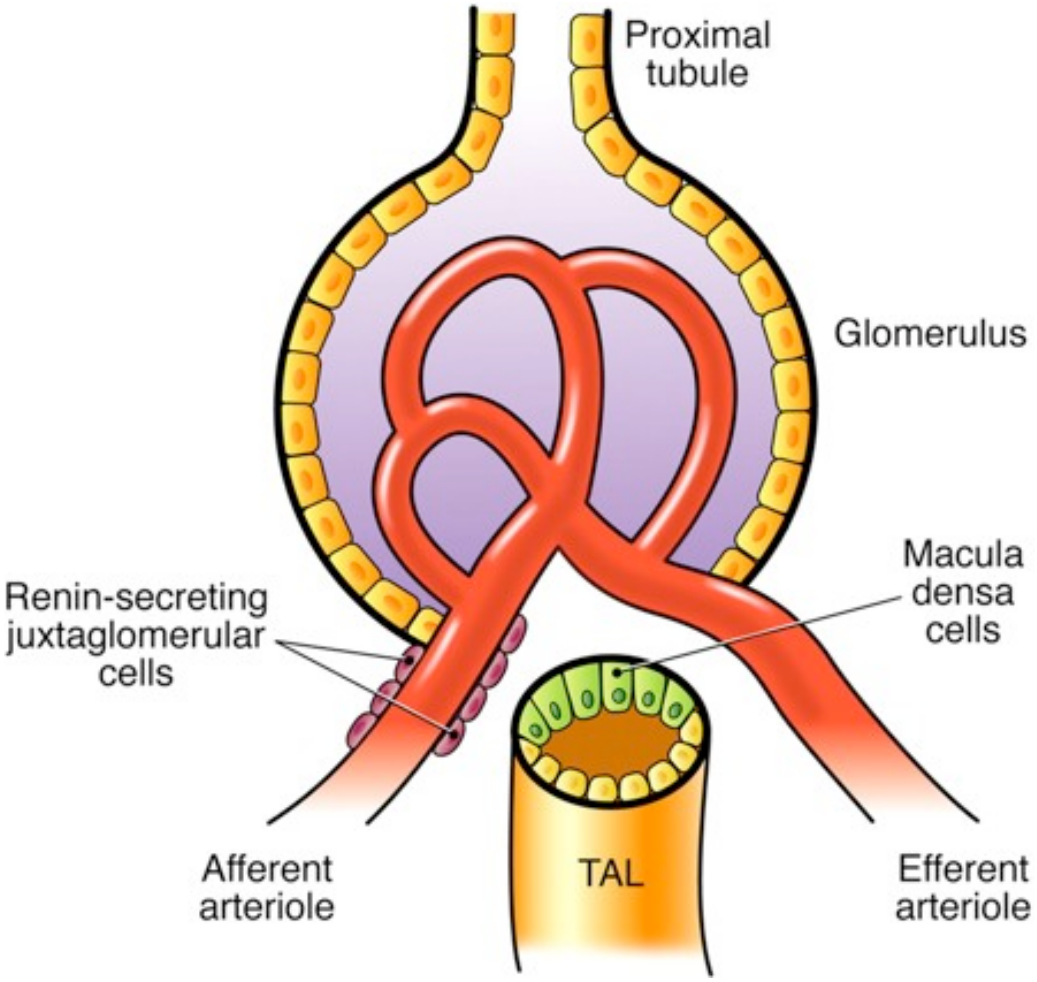


Figure. 1.2 Structure of the glomerulus, indicating the juxtaposition of the granular juxtaglomerular cells of the afferent arteriole and the epithelial cells of the macula densa in the same nephron. Taken from Mullins *et al*., 2006

1.3.1.2.2 Myogenic autoregulation

Another mechanism of RBF and GFR regulation is the ability of individual blood vessels to respond to changes in wall tension, occurring in response to alterations in arterial pressure. This activates a vascular sensor element that regulates vascular smooth muscle tone. Existence of an autoregulatory activity when the TGF response is blocked supports a contributory role of the myogenic mechanism in the autoregulatory responses of the renal vasculature (Takenaka *et al*., 1994).

1.3.2 Renal sodium transporters

Renal sodium handling is vitally important for body fluid maintenance and the long-term regulation of blood pressure. Along with renal haemodynamics, renal sodium handling largely depends on the regulation of renal tubular transporters. Sodium is freely filtered at the glomerulus and subsequently 99% of the filtered sodium is reabsorbed by the combined action of ion channels, ion exchangers and ion transporters along the nephron. A brief overview of sodium transport in the individual nephron segments is given below.

1.3.2.1 Proximal tubule

The proximal tubule is the first part of the nephron leading from the Bowman's capsule to the loop of Henle and lies in the cortex of kidney (Fig. 1.2A). About 65% of filtered sodium is reabsorbed by the proximal tubule. The apical membrane of the proximal tubule is enriched with at least 20 different sodium co-transporters coupled with organic substrates such as amino acids and carbohydrates. Nevertheless, these transporters collectively mediate only 10% of the proximal tubule sodium reabsorption (Greger, 2000). The rest of sodium transport in the proximal tubule mainly occurs by a counter-transport mechanism, the sodium-hydrogen exchanger (NHE3) (Wakabayashi *et al*., 1997). This exchanger reabsorbs sodium whilst secreting hydrogen ions (Fig. 1.2B). The driving force for sodium reabsorption

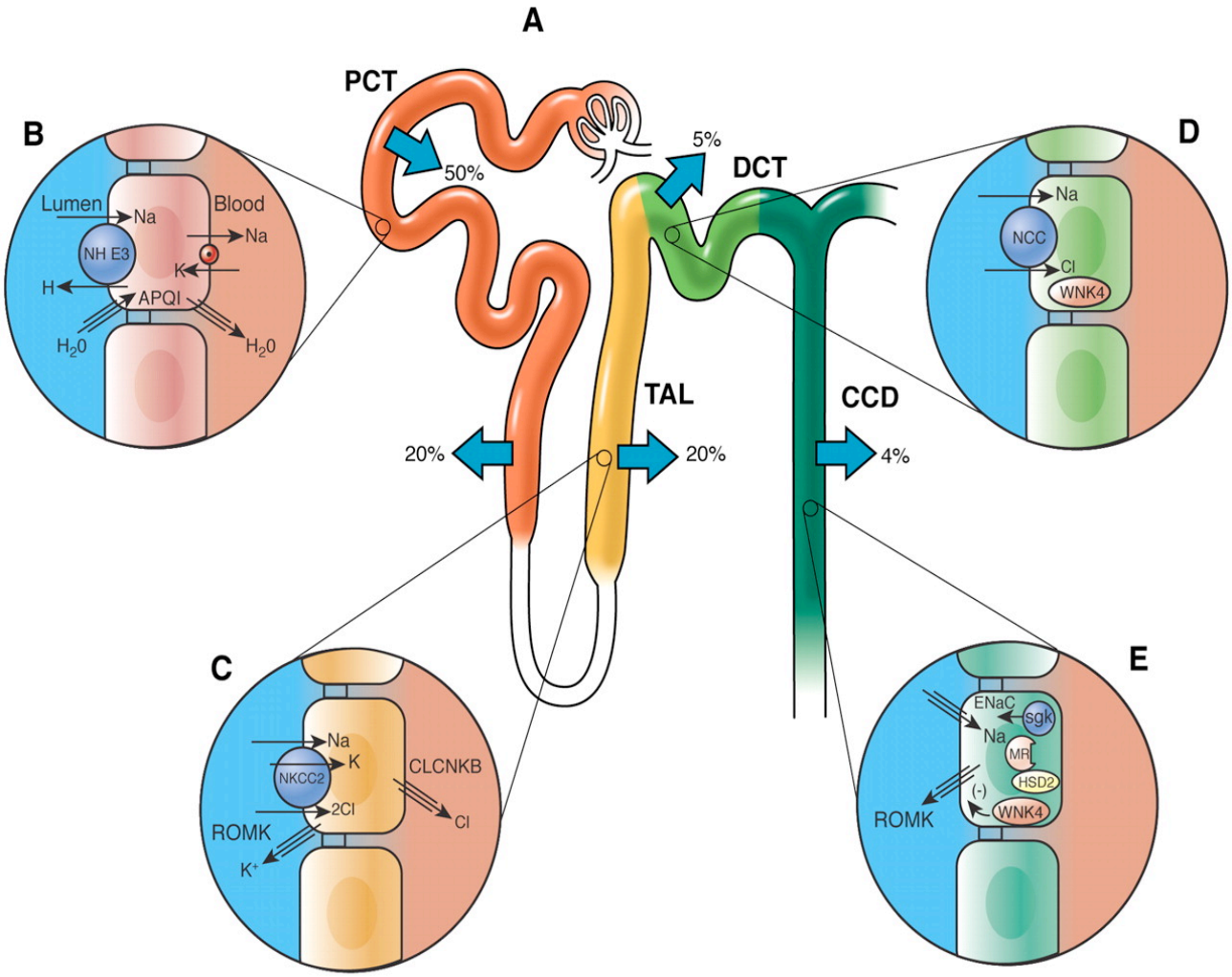


Figure. 1.2 Renal sodium transport mechanisms. A: percentage sodium reabsorption over the length of the nephron. Principal mechanisms of sodium reabsorption are shown in the proximal tubule (B), the thick ascending loop of Henle (C), the distal convoluted tubule (D), and the collecting duct (E). Taken from Mullins *et al.*, 2006.

in the proximal tubule, as for all other nephron segments, is created by the basolateral (Na^+-K^+)-ATPase (Skou, 1992). This pump extrudes 3 Na^+ and takes up 2 K^+ for each ATP hydrolyzed. This sodium potassium ATPase pump creates a concentration gradient, which causes sodium to move into the cell.

1.3.2.2 Loop of Henle

The loop of Henle consists of three distinct sections: the thin descending limb, the thin ascending limb and the thick ascending limb. About 25-30% of filtered NaCl and 20% of filtered water is reabsorbed at this nephron segment. Water reabsorption mainly occurs through the highly permeable thin descending limb. In contrast, both the thin and thick ascending limbs are virtually impermeable to water. Sodium is mainly reabsorbed through the thick ascending limb, coupled directly or indirectly to the activity of Na-K-2Cl cotransporter (Fig. 1.2C) (Greger, 1985). As in the proximal tubule, the basolateral sodium-potassium ATPase pump maintains a low intracellular sodium concentration and provides a favourable gradient for the movement of sodium from the lumen into the cell. Efficient operating of NKCC2 requires K^+ to recycle across the apical membrane through a K^+ channel (ROMK) and chloride to exit basolaterally through a chloride channel (CLCNKB) (Hebert, 2005). The potassium recycling creates a lumen-positive electrical potential gradient, which drives the reabsorption of cations through the paracellular pathway (Hebert, 2005). The TAL also has a sodium-hydrogen counter-transport mechanism like the proximal tubule, which also mediates sodium reabsorption and hydrogen secretion.

1.3.2.3 Distal convoluted tubules

About 5% of filtered sodium is reabsorbed in this part of the nephron. The sodium reabsorption processes are driven by the basolateral (Na^+-K^+)-ATPase. This pump maintains a low sodium concentration inside the cell which favours Na^+ uptake across the apical membrane by the Na^+Cl^- cotransporter (Fig. 1.2D) (Gamba *et al*., 1993). Na^+ is pumped out by the (Na^+-K^+)-ATPase and Cl^- leaves the cell via the basolateral KCl symporter. Sodium reabsorption is also mediated by the sodium-hydrogen exchange (NHE2), but to a lesser extent.

1.3.2.4 Connecting tubules and collecting duct

The transport of sodium in the connecting tubule and collecting duct is responsible for final adjustment. This site reabsorbs only 4% of the total filtered sodium. The absorptive process again depends on the basolateral (Na^+-K^+)-ATPase. Na^+ enters the cell via Na^+ channels, epithelial Na^+ channels (ENaC), along its electrochemical gradient (Fig. 1.2E) (Garty & Palmer, 1997). The Na^+ current through these channels causes a strong depolarization of the apical membrane. Due to this depolarization the cellular potassium reaches above its equilibrium concentration. Thus K^+ leaves the cell through the ROMK-type K^+ channels (Schlatter *et al*., 1993).

1.4 Role of angiotensin II on blood pressure regulation

Angiotensin II acts on multiple sites in the body such as the kidney, brain, adrenal and vascular smooth muscle. Through its combined effect on these sites the RAAS plays a key role in fluid and electrolyte balance and regulation of blood pressure.

1.4.1 Heart

It has been shown in several studies that ACE inhibition has a significant role in the treatment of heart failure (Swedberg & Kjekshus, 1988), myocardial ischaemia (Elfellah & Ogilvie, 1985; van Gilst 1986) and hypertension (Johns *et al*., 1980; Baker *et al*., 1980). Upon binding with AT_1 receptors, ANG II has effects on the regulation of heart rate, contractility and cell growth. ANG II-induced positive chronotropic effects have been demonstrated in dogs (Kobayashi *et al*., 1978) and rats (Li *et al*., 1996) and other experimental animals. A marked suppression of the chronotropic action is observed following AT_1 receptor blockade, however β-adrenoceptor antagonists do not have any influence on this effect. This confirms that the positive chronotropic action of angiotensin II is directly attributable to AT_1 receptor activation and not secondary to release of catecholamines from sympathetic nerve terminals within atrial tissues. In the long-term, ANG II exerts cytokine-like effects which promote cell growth and migration, extracellular matrix deposition, and vascular and electrical remodelling (Baker *et al*., 1992).

1.4.2 Brain

Angiotensin II acts at several sites in the central nervous system to regulate blood pressure. It has been demonstrated that angiotensin II exhibits its regulatory role on blood pressure by modulating autonomic nervous system activity (Fink, 1997), the hypothalamic-pituitary-adrenal axis (Aguilera & Kiss, 1996) and baroreflex control (Averill & Diz, 2000). In addition, AT_1 receptor activation induces vasopressin secretion (Aguilera & Kiss, 1996) in the brain and ANG II also stimulates thirst and sodium appetite (Fitzsimons, 1998). Furthermore ANG II is involved in the regulation of neurotransmitters such as noradrenaline and 5- hydroxytryptamine (5-HT) and inhibits acetylcholine release (Phillips & Sumners, 1998).

Activation of sympathetic nerves reflects both enhanced neurotransmitter release at nerve terminals and increased synaptic transmission through sympathetic ganglia (Reid, 1992). Since ANG II does not cross the blood brain barrier, circulating ANG II can only influence central processes through interaction with receptors in circumventricular areas of brain that lack the blood brain barrier (Paton *et al*., 2008). It is postulated that such binding promotes the transmission of nerve signals to other brain regions that lie behind the blood-brain barrier, thereby exerting effects such as stimulating the release of vasopressin from the posterior pituitary and also drinking behavior. (Unger *et al*., 1988) It is well established that along with the actions of peripheral RAS components in certain regions of the central nervous system (CNS), an independent RAS also exits in the brain (McKinley *et al*., 2003). It has been shown that a double transgenic mice expressing hAGT under a brain-specific promoter and a systemically expressing hREN, were reported to have a 15mm Hg

increase in blood pressure (Morimoto *et al*., 2001). The combined action of both circulating and brain RAS is responsible for increased sympathetic nerve activity during increased ANG II levels.

1.4.3 The Adrenal Gland

ANG II is a principle regulator of aldosterone biosynthesis in the zona glomerulosa of the adrenal cortex. ANG II can act acutely to stimulate aldosterone biosynthesis and chronically to increase the capacity of the adrenals to produce aldosterone (Müller J. 1995). In acute stimulation, ANG II increases cholesterol availability to the inner mitochondrial membrane by the activation of steroidogenic acute regulatory (StAR) protein. ANG II stimulates the StAR both in vitro (Cherradi *et al*., 1997, Betancourt-Calle *et al*., 2001) and invivo (Peters *et al*., 1998). Following the translocation of cholesterol to the inner mitochondrial membrane, ANG II also stimulates the expression of enzymes which are involved in subsequent steps of aldosterone synthesis such as cholesterol side-chain cleavage (Bird *et al*., 1996). ANG II also catalyzes the conversion of corticosterone to aldosterone by stimulating the transcription of CYP11B2 (Muller 1995). Chronic stimulation by ANG II results in zona glomerulosa (ZG) hypertrophy and hyperplasia increased CYP11B2 and consequently increased the capacity of the adrenals to produce aldosterone (Connell *et al*., 2008).

At the cellular level the stimulatory action of ANG II on aldosterone production is exerted mainly through the AT_1 receptors of ZG. This causes phospholipase C to stimulate intracellular production of 1,4,5 inositol triphosphate and 1,2-

diacylglycerol which, in turn, activate protein kinase C. AT_1 receptor binding also activates calcium/calmodulin-dependent kinases (CaMK), and mitogen-activated protein kinase (MAPK, Hunyady & Cat, 2006). Activation of these signalling cascades, leading to the release of Ca^{2+} from the endoplasmic reticulum (ER) and subsequent cell membrane depolarization with additional flow of extracellular Ca^{2+} into the cytoplasm. These events culminate with elevated aldosterone production by stimulating the early and the late regulatory steps in aldosterone production (Lemarié et al., 2008). Aldosterone secretion is also regulated by adrenocorticotropic hormone (ACTH). ACTH secretion is in turn regulated through ANG II-mediated activation of the central nervous system (Unger *et al*., 1988).

1.4.4 The Kidney

ANG II is one of the most potent sodium retaining hormones in the body. Sodium retention results from the interplay between renal haemodynamic control and direct actions on renal tubular sodium transport. In addition, extra-renal mechanisms, specifically activation of renal sympathetic nerves and stimulation of aldosterone secretion, contribute to the effects of ANG II on sodium balance and thus blood pressure regulation.

1.4.4.1 Role of ANG II on the regulation of renal haemodynamics

In the kidney ANG II constricts both the afferent and efferent arterioles of the glomerulus (Arendshorst *et al*., 1999; Navar *et al*., 1996). ANG II affects both arterioles with similar potency, although some studies indicate a preferential

constriction of the efferent arterioles (Carmines & Navar, 1989). The relatively greater constriction of the efferent arteriole in response to ANG II serves to maintain a proportionally higher glomerular hydrostatic pressure than would be the case if both arterioles responded to the same degree. Thus there is a smaller reduction of GFR compared to larger decreases in renal blood flow and the filtration fraction rises (Carmines *et al*., 1987). The reduction in GFR is also caused by a reduction in glomerular filtration coefficient by ANG II. Glomerular filtration coefficient is influenced by the changes in contractility of mesangial cells caused by ANG II receptor binding (Paul *et al*., 2006; Sharma *et al*., 1998). Along with its direct effects, ANG II also stimulates the release of several vasoactive components such as nitric oxide, arachidonic acid metabolites and endothelin. The vasoconstrictory action of ANG II is associated with increases in cytosolic calcium and enhanced phospholipase A_2 activity. These in turn stimulate the action of endothelial NO synthase and cyclooxygenase. As a result, the constrictor actions of ANG II are, to a degree, offset by the vasodilatory action of these vasodilators (Zou *et al*., 1998). NO buffers the action of ANG II with a greater potency on afferent arterioles again preserving hydrostatic pressure and GFR (Ito *et al*., 1993). Another modulator of ANG II-mediated vasoconstriction is endothelin 1 (ET1). ET1 gene over expression and synthesis in vascular smooth muscle cells is augmented by ANG II (Dohi *et al*., 1992; Rossi *et al*., 1999).

In addition to direct constriction of the glomerular arterioles and mesangium, ANG II also modulates the sensitivity of the TGF mechanism. (Navar *et al*., 1996; Paul *et al*., 2006) Studies in AT1R null mice indicate that ANG II does not mediate TGF

(Schnermann *et al*., 1997). Nevertheless, several studies have provided evidence that the level of activity of ANG II modifies the sensitivity of the vascular and mesangial elements to macula densa feedback (Mitchell *et al*., 1992; Schnermann *et al*., 1997; Traynor *et al*., 1999). An enhanced TGF response is found during exogenous ANG II infusion. (Mitchell & Navar, 1988). Conversely, ANG II antagonist or ACE inhibitors and salt loading attenuates TGF (Bramm *et al*., 1995; Ploth, 1983; Schnermann, 1998b). It has also been demonstrated that TGF responses are markedly attenuated in both AT_1 receptor knockout and ACE-deficient mice (Scharmann *et al*., 1997; Traynor *et al*., 1999). The enhanced sensitivity of TGF by ANG II is mediated by the stimulation of macula densa sodium chloride transport. The synergistic actions of increased ANG II to increase tubular reabsorption rate and enhanced TGF sensitivity cause a sustained reduction in distal nephron volume delivery, which maintain sodium balance more effectively to regulate blood pressure (Kobori *et al*., 2007).

1.4.4.2 Role of ANG II on tubular sodium transport

1.4.4.2.1 Role of ANG II on proximal tubule sodium transport

Considerable evidence suggests that ANG II increases proximal tubular sodium reabsorption. However it is difficult to separate out the direct effect of ANG II on tubular transport from its effect on renal haemodynamics. A rat proximal tubule microperfusion study found that ANG II stimulated proximal tubular sodium reabsorption when added to peritubular capillaries at physiological concentrations (Harris *et al*., 1977): this effect related to activation of the $(Na^+\text{-}K^+)$-ATPase (Garvin *et al*., 1991). Additionally, physiological concentrations of ANG II enhance NHE3

mediated sodium transport in the proximal tubule due to increased expression of protein (Dixit *et al*., 2004). Stimulatory effects of ANG II on sodium transport have also been found in primary cell cultures (Thekkumkara *et al*., 1998) and in kidney cortex slices from the rat kidney (Munday *et al*., 1971). In combination, ANG II, acting from either the blood or luminal side will stimulate the net reabsorption of sodium by the proximal tubule.

1.4.4.2.2 Role of ANG II on Thick Ascending Limb (TAL) sodium transport

In thick ascending limb (TAL) the apical sodium transport is mediated by two transporter proteins, Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and Na^+/H^+ exchanger (NHE3). A marked increase in both NHE3 and NKCC2 activity has been observed in response to ANG II treatment in vivo (Kwon *et al*., 2003). However a biphasic response of NKCC2 co-transport activity to ANG II has also been reported. Low concentrations (10^{-16} to 10^{-12} M) of ANG II inhibit the activity and higher concentrations (10^{-11} to 10^{-6} M) stimulate the activity in mTAL cells (Amlal *et al*., 1998). A significant increase in NKCC2 co-transporter has also been reported in rats with congestive heart failure, which have high plasma renin levels and presumably high plasma ANG II levels. Furthermore treatment of rats with congestive heart failure with the AT_1-receptor blockade normalized the abundance of NKCC2 (Staahltoft *et al*., 2002). Several lines of evidence also showed that aldosterone does not have any regulatory effect on the abundance of TAL sodium transporters (Masilamani *et al*., 1999, Neilsen *et al*., 2002). Thus ANG II regulation of NHE3 and NKCC2 abundance is directly mediated by ANG II.

1.4.4.2.3 Role of ANG II on distal convoluted tubule sodium transport

The sodium chloride cotransporter (NCC) is expressed in the apical membrane of the distal convoluted tubule. In *Xenopus* oocytes, ANG II increases NCC activation by abrogating WNK4-mediated inhibition of NCC (Kahle *et al*., 2003; Yang *et al*., 2005). This effect was prevented by AT_1 receptor blockade (San-Cristobal *et al*., 2009). This WNK-4 mediated regulation of NCC is also observed in mouse models (Lalioti *et al*., 2006; Yang *et al*., 2007). A further study suggested that ANG II induces phosphorylation of NCC at key regulatory sites through the SPAK/OSR1 kinases (San-Cristobal *et al*., 2009; Talati *et al*., 2010). In rat, an acute trafficking of NCC from the sub-apical membrane to apical plasma membrane was observed following ANG II infusion (Sandberg *et al*., 2007). Conversely the ACE inhibitor captopril reverses the trafficking. This indicates that ANG II also plays a vital role in NCC trafficking (Sandberg *et al*., 2007).

Dietary sodium restriction increases plasma aldosterone levels, which in turn increases the NCC abundance in distal convoluted tubules. A similar scenario is also observed when animals were either infused with aldosterone or oral administration of synthetic mineralocorticoid (Kim *et al*., 1998). Together, these data indicate that NCC is directly regulated by aldosterone. Dietary sodium restriction in mice increases renal NCC expression and its phosphorylation at Thr53, Thr58 and Ser71. However this response is attenuated in the SGK1 knockout mouse. This indicates the contributory role of SGK1 on the regulation of NCC expression and phosphorylation by dietary sodium restriction (Vallon *et al*., 2009).

1.4.4.2.4 Role of ANG II in connecting tubule and collecting duct sodium transport

The amiloride sensitive sodium channel, ENaC, is localized in the connecting tubule and the collecting duct and plays a vital role in the regulation of renal sodium excretion and blood pressure regulation (Lifton *et al*., 2001). ENaC is a hetero-oligomer consisting of three subunits, namely α,β and γ (Canessa *et al*., 1994). Among these subunits, the α subunit is considered as the rate limiting subunit for assembly of the mature ENaC complexes (Beutler *et al*., 2003; May *et al*., 1997). Aldosterone is one of the most important regulators of ENaC; it strongly up regulates the abundance of αENaC, both at the protein and mRNA levels (Stokes & Sigmund, 1998). Elevated abundance of αENaC was observed when rats were placed on either dietary sodium restriction or infused with aldosterone (Masilamani *et al*., 1999). The abundance of β and γ subunits was unchanged indicating the selective induction of αENaC by aldosterone. Immunofluorescence study showed a redistribution of ENaC to the apical region of the collecting duct principal cells in these animals (Butterworth, 2010). Western blot analysis also revealed that aldosterone induced a shift in molecular weight of αENaC from 85 kDa to 70 kDa which might be responsible for the activity of the channel by increasing the open probability (Masilamani *et al*., 1999).

Several studies have shown that the serum glucocorticoid- inducible serine/threonine kinase (SGK1) has a regulatory role in the control of ENaC-mediated sodium transport (Chen *et al*., 1999; Naray-Fejes-Toth *et al*., 1999). Coexpression of the SGK1 significantly enhances the activity of ENaC heterologously expressed in *Xenopus* oocytes (Chen *et al*., 1999; Naray-Fejes-Toth *et al*., 1999) and A6 cells

(Faletti *et al*., 2002). SGK1 mediated activation of ENaC is partly controlled by phosphorylation of Nedd4. Nedd4 is an ubiquitin protein which interacts with ENaC, causing ubiquitination and degradation of the channel complex. Phosphorylation of Nedd4 by SGK1 diminishes its binding affinity for the target protein and thus disrupts the ubiquitination of ENaC (Debonneville *et al*., 2001; Eaton *et al*., 2010; Snyder *et al*., 2002). This causes an increase in ENaC protein abundance in the cell membrane (Debonneville *et al*., 2001). Thus inhibition of ENaC degradation by SGK1 is considered to participate in the regulation of renal sodium excretion by aldosterone.

Although aldosterone plays a vital role controlling the sodium transport in the distal nephron, several studies have also identified a direct contribution of ANG II in these segments. (Peti-Peterdi *et al*., 2002; Wang & Giebisch, 1996). In isolated perfused CCD segments dissected from rabbit kidneys, ANG II markedly increased the apical entry of sodium. This effect was blocked completely by the specific ENaC channel blocker benzamil (Peti-Peterdi *et al*., 2002). Moreover, it has also been shown that AT_1 receptor blockade markedly decreased the abundance of αENaC in NaCl restricted rats. Conversely αENaC abundance was significantly increased in rat kidney cortex by systemic infusion of angiotensin II (Beutler *et al*., 2003). In summary ANG II regulates the sodium absorption by the kidney in part by AT_1 receptor-mediated regulation of the abundance of αENaC. Furthermore, a targeted proteomics approach in AT_{1a} receptor knockout mice has revealed that during dietary NaCl restriction the abundance of αENaC and NCC were markedly decreased in knockout mice compared to wild type mice, although circulating aldosterone levels

in knockout mice were significantly higher (Brooks *et al*., 2002). This indicates that ANG II directly plays an important role in the regulation of Na^+ transporter and channel proteins in the "post-macula densa" region of the renal tubule independent of altered circulating aldosterone concentrations.

1.5 Mutations in renal sodium transporters cause blood pressure abnormalities/disorders

Mutations in the genes encoding renal sodium transporters, or in genes that influence the activity of renal sodium transporters, have been linked to electrolyte and blood pressure disorders in humans (Lifton *et al*., 2001). Modelling of these defects in mice provides a useful tool to study the physiological relationship between renal sodium homeostasis and blood pressure control (Schnermann, 2001). Sodium transporter defects, for example, can cause a permanent reduction in extracellular fluid volume which in turn stimulates renin release and aldosterone synthesis. These changes cause an activation of Na^+ conservation mechanisms such as adaptation by the increased transporter activity in the downstream nephron segment. In most of the cases the sodium balance is restored and a steady state is achieved. But when sodium balance restoration fails it causes salt wasting and volume depletion and even death.

1.5.1 Proximal tubule transporters

As discussed earlier NHE3 is the major transporter in the proximal tubule which accounts for 50-60% of sodium reabsorption. However NHE3 knockout mice exibit only a mild volume depletion and hypotension (Schultheis *et al*., 1998; Wang *et al*., 1999). It has been shown that NHE3 deletion results in the delivery of more sodium to the MD, which causes a reduction in blood flow and GFR by resetting the TGF (Lorenz *et al*., 1999). The loss of NHE3 is also compensated by enhanced expression of the Na-phosphate cotransporter, and the collecting duct γ-ENaC subunit (Brooks *et al*., 2001). Up-regulation of NHE2 in the early distal tubule has also been demonstrated (Bailey *et al*., 2004) and these changes may well reflect significant activation of the RAS. NHE3 null mice actually show an increased, rather than diminished, tubular sodium reabsorption (Bailey *et al*., 2004), which illustrates the redundancy of sodium transport within the kidney.

Similarly, deletion of aquaporin 1 (aqp1) which causes impaired proximal tubular sodium reabsorption, was found to result in moderate hypotension (Schnermann *et al*., 1998a). Proximal impairment is compensated by activation of TGF. To understand the individual role of TGF and distal sodium reabsorption the Aqp1 -/- mouse was crossed with the adenosine $A1_a$ receptor knockout mouse, which has a defective TGF. Surprisingly, the double knockouts maintained the sodium balance (Hasimoto *et al*., 2004). This indicates the importance of distal tubular sodium reabsorption in sodium balance and may explain why no severe blood pressure defects associated with impaired proximal tubule function have been identified in humans.

1.5.2 Thick Ascending Limb (TAL) transporters

Genetic defects in any of the TAL transporters such as NKCC2, ROMK, CLCNKB cause Bartter's syndrome. It is characterized by severe polyuria, low plasma potassium, alkalosis, hypercalciuria, proteinuria and low blood pressure (Hebert, 2003). Knockout mice for NKCC2 die within 2 week of birth from severe volume depletion, which demonstrates the importance of the transporter in fluid balance (Takahashi, 2000). Mouse Kcnj1 knockouts represent Bartter's syndrome caused by a null mutation in the ROMK gene (Lu *et al*., 2002). Similar to NKCC2 knockout mice, the majority of null mutants die within 3 week of age. In spite of reductions in GFR the survivors had marked renal sodium loss. This may be due to an inability to correct renal sodium handling by TGF. Thus K^+ channels are required for efficient sensing of luminal sodium by MD cells (Vallon, 1997).

1.5.3 Distal convoluted tubule transporter

Loss-of-function mutations in the gene encoding NCC at the distal convoluted tubule cause Gitleman's disease. This disease is characterized by hypokalemia, metabolic alkalosis and mild hypotension. Mice lacking NCC represent the animal model for Gitleman's disease and do not show any salt wasting phenotypes (Schultheis *et al*., 1998), since the function of NCC is compensated by an increased abundance of the γ-subunit of ENaC (Brooks *et al*., 2001). Another autosomal dominant disorder called Gordon's syndrome, characterized by hypertension and hyperkalemia, is also linked with increased NCC activity since thiazide treatment can correct this disorder (Wilson *et al*., 2001). However there is no significant linkage between Gordon's

syndrome and the NCC gene locus (Gamba, 2005). It has been observed that independent mutation in two serine-threonine kinase family WNK4 (Wilson *et al*., 2001) and WNK1 (Newhouse *et al*., 2005) is responsible for this disorder. The mutations that cause Gordon's disease produce a gain-of-function for WNK1 or loss-of-function of WNK4 (Flatman, 2007). Both WNK1 and WNK4 modulate the NCC activity by phosphorylation through SPAK and ORS1 kinases (Richardson *et al*., 2008; San-Cristobal *et al*., 2009).

1.5.4 Connecting tubule and collecting duct transporters

Loss-of-function mutations in any of the three ENaC subunits leads to pseudohypoaldosteronism type I, which results in sodium wasting, hypotension, hyperkalemia and high mortality immediately after birth (Chang *et al*., 1996; Strautnieks *et al*., 1996). Several gene inactivation studies for the individual three subunits of ENaC identified their role in sodium reabsorption and survival. Mice with α-ENaC knocked out die within 40 hour of birth from failure to clear their lungs (Hummler *et al*., 1996). β-ENaC and γ-ENaC null mutants exhibit early renal dysfunction that leads to death from dehydration and hyperkalemia shortly after birth (Barker *et al*., 1998; McDonald, 1999). Sodium reabsorption defects in the terminal nephron cause severe salt wasting since no other mechanisms are able to compensate for them. This results in severe volume depletion and death.

Liddle's syndrome (Liddle *et al.,* 1983) is a monogenetic form of arterial hypertension caused by a gain in function mutation or deletion of ENaC. It is caused by specific mutations at the conserved PY motif in either β- or γ-ENaC. Ubiquitin

ligases, such as Nedd4-2, interact with the PY motif, leading to ubiquitination and degradation. The mutation in the PY motif rescues the ENaC subunits from degradation or internalization by Nedd4-2 (Kamynina *et al*., 2001). This leads to an increase in ENaC activity, and increased water and salt reabsorption in the renal collecting tubules (Schild *et al*., 1996).

1.6 The mRen2 model of hypertension

It is well recognized that the renin-angiotensin system is involved in the regulation of blood pressure and fluid homeostasis. This enzyme cascade is one of the well characterized systems that contribute to the pathogenesis of polygenetic essential hypertension. Several experimental animal models have been developed to explain the hypertensionogenic mechanism mediated by the renin-angiotensin system. Among them the two-kidney-one-clip (2K1C) Goldblatt (Goldblatt *et al*., 1934) and chronic ANG II infused hypertensive rats the most frequently used ANG II-dependent models of hypertension. Gene targeting approach has also been used extensively to define the precise roles of RAS components in the genesis of hypertension (Mullins *et al*., 2006). It provides the opportunity to study the effect of precise molecular genetic intervention to study the complex system of blood pressure regulation in whole animal. Several animal models have been developed in the last 20 years to study the contributory role in the development of hypertension. In the current study an inducible hypertensive transgenic rat with the mouse Ren2 gene (Kantachuvesiri *et al*., 2001) has been used.

1.6.1 Renin locus

In human and rat only one renin gene is present but mice have two genotypes at the renin locus. Some inbred mouse strains such as C57BL/6 and BALB/c contain one renin gene (Ren-1^c) and are analogous to human and rat. Other strains such as SV129 and DBA are unique in having two renin genes termed Ren-1^d and Ren-2 (Field & Gross, 1985). The Ren-1^d and Ren-2 genes are closely related and this is thought to arise from a gene duplication event that occurred about three million years ago (Dickinson *et al*., 1984). The Ren-1^d genes express renin predominantly in the juxtaglomerular apparatus of the kidney, whereas the main site for the Ren-2 gene expression is submaxillary gland and also in the kidney at very low levels (Sigmund & Gross, 1991). All three mouse renin genes share the same overall genomic organization and translate into highly homologous proteins. However the product of Ren-2 lacks putative consensus sites for asparagine-linked glycosylation. The characteristic glycosylation of the Ren-1^d protein may be related to renin secretory granule formation in JG cells and maturation of pro-renin. (Clark *et al*., 1997; Mullins *et al*., 2000).

Targeted disruption of the Ren-1^c gene in single renin gene strains has been reported to result in a reduction in blood pressure (Takahashi *et al*., 2005). These mice also show undetectable plasma renin, ANG I and ANG II, reduced urinary aldosterone and renal morphological changes. Several gene targeting studies have been performed to identify individual contributions of the Ren-1^d and Ren-2 genes to the maintenance of BP in two renin gene carrying mice (Bertaux *et al*., 1997; Clark *et al*., 1997; Sharp *et al*., 1996; Pentz *et al*., 2001). It has been demonstrated that targeted disruption of the Ren-1^d gene in two renin gene carrying mice causes a

reduction in blood pressure in female mice along with a lower plasma renin concentration but not in male animals (Clark *et al*., 1997). This indicates that the production of renin from Ren-2 is adequate to compensate the loss of Ren-1^d in male mice which express larger amounts of Ren2 in the submandibular gland but could not fully compensate in female mice. In contrast, another Ren-1^d gene disruption study showed a significant reduction in plasma active renin concentration accompanied with reduced blood pressure in both sexes (Pentz *et al*., 2001). Another $Ren1^d$ deletion strain was reported to have no change in blood pressure, suggesting that the product of the Ren-2 gene is physiologically active and can compensate for the loss of Ren-1^d (Bertaux *et al.*, 1997). Conversely no change in BP was observed in adult mice homozygous for the mutated Ren-2 gene in mice carrying two renin gene (Sharp *et al*., 1996). It has been shown that the presence of two renin genes on a C57BL/6J genetic background resulted in a higher baseline BP compared to the wild type C57 mice carrying a single renin gene (Wang *et al*., 2002). Mice with two renin genes exhibited 100-fold higher PRA and PRC values, indicating the contribution of over activity of the renin-angiotensin-aldosterone cascade. Furthermore, AT_1 receptors blockade showed a marked attenuation in BP in two- but not in one-renin gene mice. These observations suggest that the Ren-2 gene is physiologically active in two-renin gene mice and participates in the increase in BP under basal conditions.

1.6.2 TGR(mRen2) transgenic rat

To further assess the role of an additional renin gene, the Ren2 gene was introduced in mice carrying a single renin gene. The mouse model transgenic for Ren-2 gene did not cause hypertension due to species-specific interaction of the renin-angiotensin system. Conversely an increased blood pressure was observed when the renin

derived from the submaxillary gland of mouse (product of Ren2 gene) was injected into rats (Onoyama *et al*., 1978). As mouse renin is capable of cleaving rat angiotensinogen to produce angiotensin I and increases blood pressure if injected into rats, a transgenic rat was created by introducing the mouse Ren2 gene into the rat genome (Mullins *et al*., 1990). This transgenic rat model has given the opportunity to study hypertension in which the genetic basis of the disease is known. This was the first transgenic rat in hypertension research.

These transgenic rats, TGR(mRen2)27, developed fulminant hypertension by 8 weeks of age (Mullins *et al*,. 1990). The homozygotes had higher blood pressure than heterozygotes, indicating that the hypertensive phenotype depends on the dose of transgene. Blood pressure levels also differ between male and female rats, both in heterozygous and homozygous strains (Lee *et al*., 1996). Furthermore both ACE inhibition and AT_1 receptor blockade effectively lowered the blood pressure, indicating that the hypertension is ANG II-dependent (Bader *et al*., 1992, Mullins *et al*., 1990). The hypertensive phenotype of this model is characterized by suppressed circulating renin, ANG I and ANG II. The mRen-2 expression is found in the adrenal gland, followed by the thymus, brain, gastrointestinal, urinary tracts, kidney and lung (Engler *et al*., 1998, Mullins *et al*., 1990). This indicates the role of local RAS as the cause of hypertensive phenotype in these rats. But the low mRen-2 expression in the kidney suggests the major involvement of extrarenal RAS in this model such as adrenal RAS (Sander *et al*., 1992). However, the aldosterone inhibitor spironolactone did not decrease blood pressure, which suggests that other factors must be responsible for hypertension in this model (Sander *et al*., 1992).

1.6.3 Inducible Cyp1a1-mRen2 transgenic rat model

The experimental animal models that have been used to study angiotensin II-dependent hypertension require surgical or pharmacological manipulation or depend on the constitutive expression of heterologous transgene to induce angiotensin II-dependent hypertension. To overcome these problems and to get a tightly temporally regulated gene expression to induce hypertension, an inducible transgenic rat was generated by inserting a mouse renin gene under the control of cytochrome P450 promoter, Cyp1a1 (Kantachuvesiri *et al*., 2001). The Cyp1a1 promoter was placed

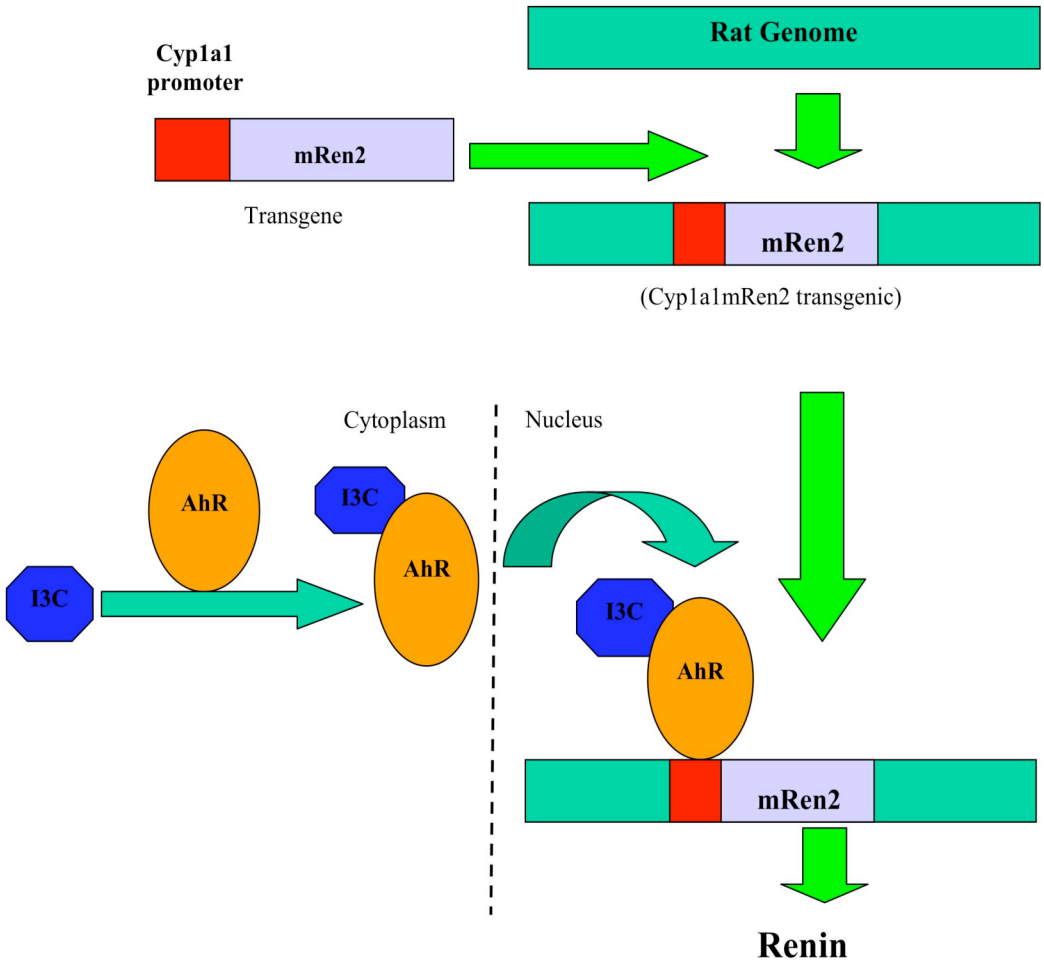


Figure 1.3: Schematic presentation of the transgene induction in Cyp1a1-mRen2 transgenic rat by I3C.

upstream of mRen-2 and introduced into Fischer F344 single cell embryos by microinjection. The normotensive transgenic line was considered as transgene insertion in neutral genomic site. Further analysis by southern blot and *in situ* hybridization identified the insertion of the transgene in the Y chromosome. The inducible promoter Cyp1a1 catalyses the oxidation of a variety of xenobiotic compounds such as indole-3-carbinol (I3C) (Campbell *et al*., 1996; Forrester *et al*., 1992; Smith *et al*., 1995). This promoter is not constitutively expressed; rather is highly inducible upon exposure to various aryl hydrocarbons. Initially aryl hydrocarbon compounds bind with the aryl hydrocarbon receptor (AhR) which is a basic helix-loop-helix transcription factor (Jellinck *et al*., 1993). Upon binding, the AhR crosses the nuclear membrane and binds with specific DNA elements in the Cyp1a1 promoter (Loub *et al*., 1975; Pelkonen *et al*., 1982). This causes an initiation of expression of the Cyp1a1 promoter (as shown in Fig. 1.3). So rats carrying the Cyp1a1-mRen2 transgene do not express the mouse Ren2 gene constitutively, but it is selectively induced in the liver by dietary administration of I3C.

1.6.3.1 Hypertensive phenotype of Cyp1a1-mRen2 transgenic rat

Continuous dietary administration of I3C in transgenic male rats leads to sustained hypertension, whereas the transgene negative rats remained normotensive after I3C administration (Kantachuvesiri *et al*., 2001). Importantly, the severity of hypertension in the transgenic rats is dose-dependent (Kantachuvesiri *et al*., 2001): low-dose (0.15% w/w) dietary supplementation of I3C leads to slowly developing hypertension, whereas at a higher dose (0.3% w/w) hypertension can enter a malignant phase, which in rats is indicated by weight loss, piloerection, hunched

posture and polyuria (Mitchell *et al*., 2006). The hypertension in this model is reversible, with the termination of I3C administration resulting in the normalization of blood pressure (Kantachuvesiri *et al*., 2001). Thus this model opens the opportunity to study the molecular and cellular events occurring during the development and reversion of hypertension.

The expression of mRen2 was mainly detected in the liver and small intestine but it was not found in other tissues such as spleen, heart, kidney, lung, adrenal gland, aorta, or skin (Kantachuvesiri *et al*., 2001). After induction for 7 days, all the components of the circulating RAS were increased in transgenic rats. The plasma prorenin concentration was increased greater than 200-fold, whereas plasma renin concentration was increased two-fold. Both ANG II and aldosterone levels in plasma were increased in Cyp1a1-mRen2 transgenic rats following induction. This increased plasma RAAS level is mainly transgene-derived. Levels of PRA and ANG II remained unchanged when the AT_1 receptor was blocked during induction, suggesting that plasma renin and ANG II level is not under the control of JG apparatus-regulated renin secretion (Mitchell & Mullins, 2005). Rather it indicates that levels of renin and ANG II depend on transgene induction and are not under the control of homeostatic regulation. In the kidney although renin mRNA was markedly suppressed in transgenic rats; nevertheless a high level of intra renal ANG II is found after induction (Mitchell *et al*., 2006). However AT_1 receptor blockade prevents the I3C induced augmentation of ANG II suggesting that elevated intrarenal ANG II levels in transgenic rats are mediated by AT_1 receptor mediated uptake of circulating ANG II or receptor mediated stimulation of the intrarenal RAS (Mitchell *et al*.,

2006). Attenuation of the hypertensive phenotype by AT_1 receptor blockade indicates that AT_1 receptor activation by elevated plasma and intrarenal ANG II levels primarily mediates the development and maintenance of malignant hypertension in Cyp1a1-mRen2 transgenic rats (Mitchell & Mullins, 2005; Mitchell *et al*., 2006; Vanourková *et al*., 2006). A higher plasma and urinary aldosterone was reported in this transgenic rat following induction. However inhibition of MR failed to alleviate hypertension, suggesting that MR activation by elevated circulating aldosterone levels does not contribute markedly to the increased arterial pressure following induction of this transgenic rat. Nevertheless, alleviation of proteinuria upon MR blockage indicates the contributory role of aldosterone in renal damage (Ortiz *et al*., 2007a).

1.6.3.2 End organ damage

In various hypertensive conditions ANG II mediates the pathogenesis of renal tissue injury by inducing inflammatory mediators and immune cells (Bledsoe *et al.,* 2006; Cheng *et al*., 2003; Haller *et al*., 1997; Pu *et al*., 2005; Rodríguez-Iturbe *et al*., 2002). A differential susceptibility to vascular injury from malignant hypertension was observed in this model (Kantachuvesiri *et al*., 2001). Despite the same level of hypertension and circulating hormonal levels histological evidence of malignant hypertension was primarily observed in mesentery and heart. Hypertensive vascular injury in the kidney was observed at a later stage. Medial thickening of vessel walls of the interlobular and arcuate arteries was observed after 7 days of induction with 0.3% I3C (Kantachuvesiri *et al*., 2001). Conversely after 10 days of induction with 0.3% I3C the renal pathological changes primarily consist of inflammation and

cellular proliferation in the cortical vessels and tubulointerstitium such as myointimal hyperplasia and tubular dilation, glomerulosclerosis, along with tubulointerstitial inflammation and proliferation (Graciano *et al*., 2007). These morphological changes together with vasoconstriction of preglomerular arteries protect the glomeruli from transmission of elevated arterial pressure and maintain normal renal haemodynamics (Opay *et al*., 2006; Petterson *et al*., 2005). However prolonged induction for 14 days caused more severe hypertensive vascular damage such as fibrinoid necrosis, endarteritis obliterans of interlobular arteries and afferent arterioles (Kantachuvesiri *et al*., 2001). This severe vascular damage may result in a marked decline in renal haemodynamic function (Liu *et al*., 2009). Thus renal vascular and interstitial inflammatory changes occur during and contribute importantly to the development of ANG II-dependent malignant hypertension in Cyp1a1-mRen2 transgenic rats.

1.6.3.3 Salt sensitivity

The hypertensive phenotype following induction is characterized by unaltered renal haemodynamics (Opay *et al*., 2006; Patterson *et al*., 2005) although in some studies a significant decrease in RPF is reported (Mitchell *et al*., 2006). This indicates that Cyp1a1-mRen2 rats with malignant hypertension exhibit a marked increase in RVR and preglomerular vascular resistance preventing the transmission of systemic hypertension to the glomerular capillaries. Since GFR is not changed, there might be a role of enhanced tubular reabsorption in the maintenance of hypertensive phenotypes. However urinary sodium excretion was not markedly altered in Cyp1a1-mRen2 rats after induction (Mitchell *et al*., 2006). Thus they have an impaired pressure natriuretic response to the increased arterial blood pressure. The kidney's

inability to maintain sodium excretion in this hypertensive rat model was examined by salt loading experiments. Feeding a low-salt diet (0.04%) substantially attenuated the development of hypertension and feeding a high-salt (8%) diet exacerbated the course of hypertension (Husková *et al*., 2010). Moreover short-term activation of the Cyp1a1-mRen2 transgene induces salt-sensitive hypertension in these transgenic rats similar to the salt-sensitive hypertension developed after short-term exposure to intravenous ANG II (Howard *et al*., 2005). The blood pressure returns to normal within 10 days after the cessation of I3C induction. Subsequently feeding these animals with a high salt diet (8.0% NaCl) caused a significant increase in blood pressure. The absence of any significant change in either GFR or RPF during the development of this salt-sensitive hypertension suggests the contributory role of enhanced tubular reabsorption. It might be possible that enhanced tubular reabsorption developed during transient induction of ANG II-dependent hypertension leads to an impaired ability of the kidney to respond to elevated salt intake and thus elevates blood pressure (Howard *et al*., 2005).

1.7 Aims of the study

Inappropriate activation of the renin angiotensin system (RAS) is a major factor contributing to the development of hypertension. The synergistic action of ANG II on renal haemodynamics and tubular transport plays a key role in the development of ANG II-dependent hypertension. Accordingly the first aim of the present study was to characterize renal haemodynamics and renal sodium handling during the development of ANG II-dependent hypertension in the Cyp1a1-mRen2.F transgenic rat.

Several studies have demonstrated that ANG II stimulates the proximal and distal tubular sodium reabsorption rate. Although most of the filtered load is reabsorbed in the proximal tubule, sodium reabsorption in the distal tubule and cortical collecting duct is very critical because the fine-tuning of sodium reabsorption occurs in this region. Thus the second aim of the study was to identify the activity of the distal tubule sodium transporters responsible for the enhanced sodium retention.

Chapter 2

Materials and Methods

2.1 Animals

All experiments described were performed in accordance with the Home Office Animals (Scientific Procedures) Act 1986. All transgenic rats used in this experiment were bred at the animal facility in the University of Edinburgh. For control purposes male Fischer rats (F344) were bought commercially (Harlan, UK). All rats were given free access to water and standard commercial rat chow (Special Diet Services, Witham, Essex, UK) and maintained under controlled conditions of temperature (21 ± 1^{O} C) and humidity (50±10%) and a light/dark cycle of 12hr.

2.2 Induction of transgene

The Cyp1a1-mRen2 transgene was induced by indole 3 carbinol (I3C, Sigma-Aldrich, UK) at the rate of 100mg/kg body weight. I3C was finely powdered by using a mortar and pestle and suspended in vegetable oil (50mg/ml). I3C administration was carried out daily by gastric gavage at 11am for either 1-day, 3-days or 7-days. Control rats received vegetable oil only (200µl/100gm). During this induction animals had free access to food and water.

2.3 Surgical protocols

For the renal function experiments, the rats (250-300 gm) were anaesthetized by intraperitoneal injection of Inactin (Thiobutabarbital, Sigma, Poole, Dorset, UK; 100mg/kg, IP) and placed on a thermostatically-controlled surgical table to maintain body temperature at 37ºC. A small incision was made over the left jugular vein and then the vessel was separated from the connecting tissue. Once the left jugular vein

was prepared for cannulation, a small puncture was made and cannulated with polyethylene tubing (PE50, Portex fine bore polythene tubing, Jencons, UK) connected with a 5ml syringe containing 0.154M NaCl. This route was used to allow intravenous infusion of solutions and additional doses of anaesthetic (if required). The right carotid artery was similarly cannulated by PE50 tubing connected with a 20ml syringe containing heparin solution (40 units per ml; Fluka, UK). A tracheotomy was performed to maintain a clear airway. A supra-pubic incision was made and the bladder was exposed by blunt dissection through the abdominal wall. The bladder was catheterised with PE-90 tubing to allow timed urine collections to be made. Deknatel silk 5.0 (Teleflex Medical, NC, USA) was used as surgical suture during the experiment. At the end of surgery, the jugular catheter was connected to a syringe pump (AL2000 model, World Precision instruments, Sarasota, FL, USA) for a continuous infusion of infusate solution (120mM NaCl, 10mM $NaHCO_3$, 5mM KCl, 15 mM LiCl, pH 7.4) containing 0.5% Inulin-FITC (Fluorescence Isothiocyanate; Sigma-Aldrich, UK) and 1% p-aminohippuric acid (Sigma-Aldrich, UK) at a rate of 1ml/hour/100gm body weight. The arterial cannula was connected to a blood pressure transducer (Powerlab, ADinstruments Ltd, Chalgrove Oxfordshire, UK) for continuous blood pressure recording (10 Hz sampling rate).

2.4 Renal clearance infusion protocol

Following surgery, rats were infused intravenously (1ml/100g BWt/hr) with the infusate described earlier and allowed to stabilise for one hour. Following stabilisation, a small blood sample was collected (approximately 75µl) into two heparinised capillary tubes (Hawksley, Lancing, Sussex.UK). Immediately after collection, the capillary tubes were spun in a haematospin 1400 centrifuge (Hawksley, Lancing, Sussex, UK) and the plasma was stored at -20°C following haematocrit measurement. For the next 40 minutes a urine collection tube with a known volume of mineral oil (to stop evaporation) was placed at the end of the bladder catheter for continuous urine collection. After this, the first urine collection tube was removed and a second blood sample taken as before. At this point a bolus dose of amiloride (2mg/kg Bwt; vehicle= 2% DMSO in infusate solution, 50µl/100gm) was given via the vein and amiloride (Sigma-Aldrich, UK) also added to the infusate (2mg/kg Bwt/h) to block ENaC. A second urine collection tube was attached and urine collected for another 40 minutes. As before, after this 40 minute period the urine collection tube was removed and a small blood sample was collected. Following this, a bolus of amiloride plus thiazide (2mg/kg Bwt vehicle= 2% DMSO in infusate solution, 50µl/100gm; Sigma-Aldrich, UK) was given and both diuretics were also added to the infusate (2mg/kg Bwt/h). Thus both ENaC and NCC were blocked during this urine collection period. A final (U3) urine collection was then made as before.

To confirm the stability of renal function during this protocol and any effect of the DMSO vehicle, a time-control experiment was performed. In the time control

experiment only vehicle was added with infusate followed by a bolus injection of vehicle only. Urine and blood samples were collected as described earlier.

At the end of the experiment terminal blood (2ml) was collected in heparinised tubes (Sarstedt AG and Co, Numbrecht, Germany) and centrifuged for 5 minutes at 16000 g to separate plasma and stored at -20°C. The animal was then sacrificed by over dose of anaesthetic and the kidneys, heart adrenals and lung were collected on dry ice and stored immediately at -70°C for further analysis. A schematic diagram of the infusion protocol for this experiment is shown in Figure 2.1.

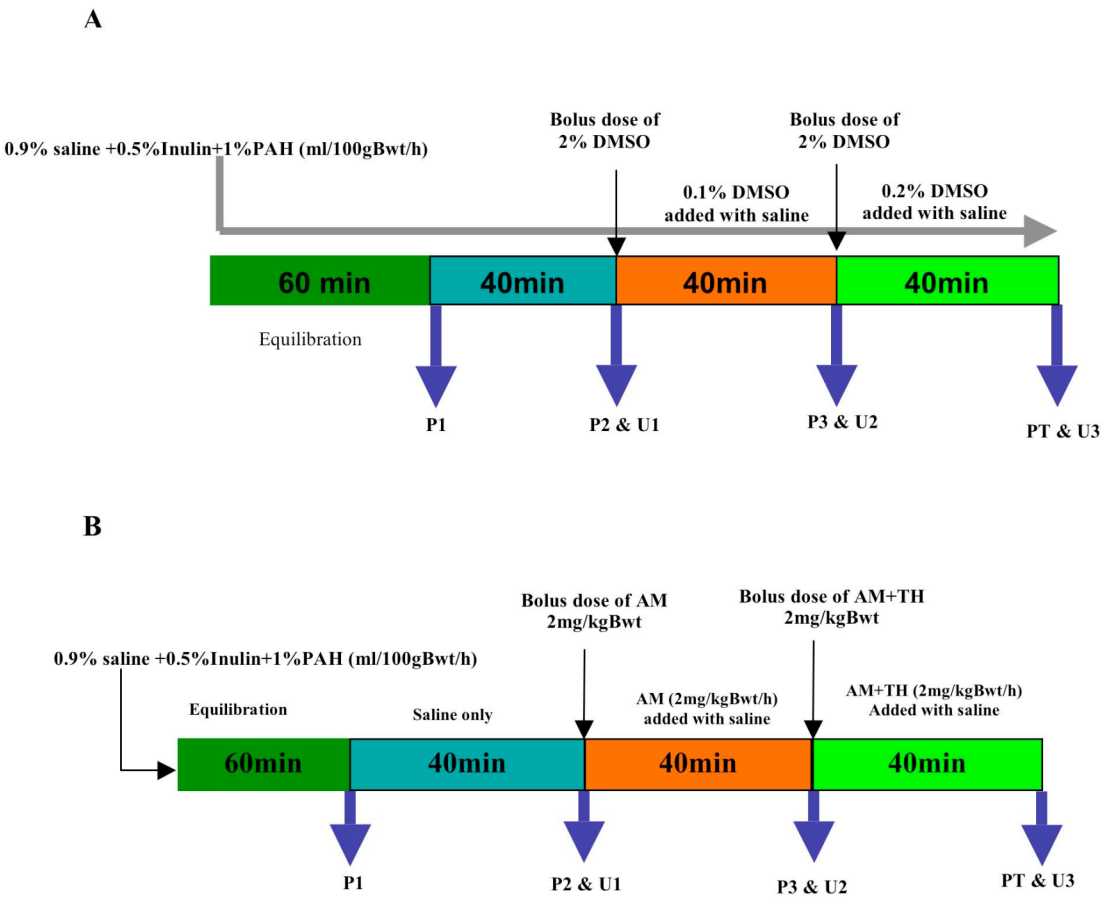


Figure. 2.1 Infusion protocol for clearance study. (A) Time control experiment, (B) Distal tubular function study.

2.5 Tail cuff plethysmography measurement of blood pressure

Systolic blood pressure (SBP) was measured using tail cuff plethysmography (tail cuff) in conscious, restrained rats (Harvard Apparatus, Edenbridge, Kent, U.K.). At the beginning, rats were conditioned to the tail cuff restraint and the warming chamber for 10-20 min/day for at least 5 days. Once a stable reading was obtained rats were considered trained and ready for the experiment. The tail cuff measures the systolic blood pressure by sensing the changes in the blood flow in the rat's tail via an optical transducer inside the cuff. Rats are placed in a heating box and warmed to around 30°C to dilate the tail artery and allow sufficient blood flow into the tail. At this point the pre-heated rat is placed inside the tail cuff restraint and a cuff was fitted on the tail. The cuff was then inflated by automatic pump, the optical transducer measures the pressure at which the blood vessel is occluded and blood flow stopped i.e. the SBP in mmHg. The data measured are amplified and viewed on a PC via a serial link, where the data are recorded from the tail cuff. A total of eight readings are taken for each measurement, four of which are on cuff inflation and four on the cuff deflation. A record is made of the four deflation values and the mean of these calculated as systolic blood pressure. The inflate values were rejected as they were usually inaccurate due to the rat reacting with movement as the cuff tightening around its tail. At least two consecutive readings were taken for each animal. However in one experiment (Figure 3-5.A) another tail cuff device (IITC Life Science, Woodland Hills, CA, USA) was used to measure mean blood pressure instead of SBP using the similar method.

2.6 Metabolic cage study

To measure fluid and electrolyte balance in conscious animals, a group of 8 transgenic rats were housed individually in metabolism cages (Techniplast, Italy) which were provided with stainless steel mesh bottoms and a urine collection pan underneath attached to a glass collection tube. Rats were kept under controlled conditions of temperature (21±1°C) and humidity (50±10%) and a light/dark cycle of 12hr. Animals had free access to powdered RM1 diet (0.3% sodium) and water. The first 3 days were considered as an acclimatization period and no data were recorded during that time. After that period, daily food consumption, water intake, urinary and faecal excretion and body weight were measured for three days of baseline recording. All the measurements were recorded at 10.00am±1 hour to avoid any effect of time. After daily collection animals were placed into a cleaned cage for the next collection. After baseline collection, animals received I3C by daily gavage for 7 days, during which measurements were made as before. Urine and faeces were stored at -4°C until required for analysis of electrolytes.

2.7 Dietary restriction of sodium

A group of transgenic rats (n=5) were prepared for measurement of SBP by the tail cuff method as described. After training, baseline SBP was measured for 3 consecutive days. Then animals were transferred to a clean home cage and fed low-sodium diet (0.03% sodium; Special Diet services, UK) for the rest of the study period. Following the 3 days of acclimatization to low sodium diet, SBP was again measured for another three consecutive days. After this animals were induced by daily gavage for seven days; SBP was measured daily during this period. At the end of the 7 days of induction renal function was measured as described above (section 2.3 & 2.4). A schematic diagram of the experimental regime for this experiment is shown in Figure 2.2.

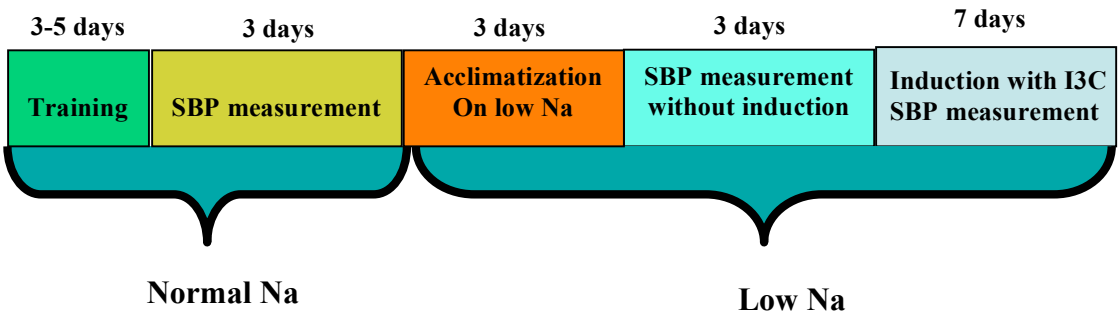


Figure 2.2: Experimental regime for dietary sodium restriction study.

2.8 Chronic inhibition of sodium chloride cotransporter

2.8.1 Baseline SBP measurement

A group of transgenic rats (n=6) maintained on standard rat chow were trained for measurement of SBP by tail cuff as described above. A baseline measurement was recorded over three consecutive days.

2.8.2 Minipump implantation

Following baseline measurement, the rats underwent surgery for subcutaneous implantation of an osmotic minipump (model 2ML1; ALZET, Cupertino, CA, USA). The pumps delivered hydrochlorothiazide (Sigma-Aldrich, UK; 4mg/kg/day in 1:1 DMSO:saline) and were primed overnight in 0.154M NaCl saline. For minipump implantation the animals were anaesthetized with isoflurane gas (2-3%) plus oxygen (0.4-0.5lit/min) and I.V. buprenorphine as an analgesic (Vetergesic, Pfizer, at 0.01ml/g body weight). The skin over the implantation site was shaved and washed. A 1 cm incision was made on the back and a haemostat was inserted into the incision to create a pocket for the pump into which a pump was inserted. The wound was closed with wound clips (Clay Adams, BD, NJ, USA) and the rats were placed into a heated recovery box until they regain consciousness. The rats were returned to their individual cage.

2.8.3 Induction and SBP measurement

Animals were allowed 24h for recovery from the effect of surgery before SBP was measured again for 3-4 days. The animals then received I3C by daily gavage for 3 days and SBP was measured during this period. At the end of induction renal function was measured as described (section 2.3 and 2.4). A schematic diagram of the experimental regime for this experiment is shown in figure 2.3.

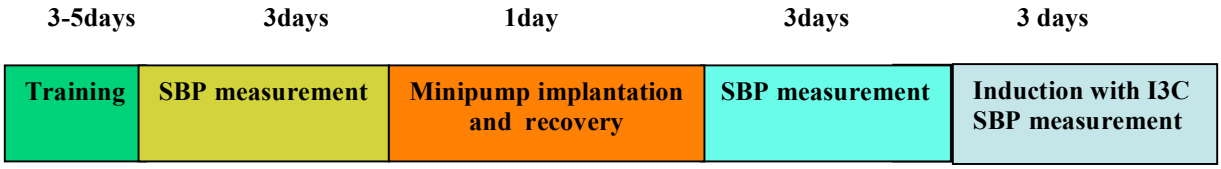


Figure 2.3: Experimental regime for chronic inhibition of NCC study

2.9 Chronic Inhibition of Mineralocorticoid Receptor

2.9.1 Baseline SBP measurement

A group of transgenic rats (n=6) maintained on standard rat chow were trained for measurement of SBP by tail cuff as described (section 2.5). A baseline measurement was recorded for three consecutive days.

2.9.2 Minipump implantation

Following baseline measurement, the animals underwent surgery for subcutaneous implantation of an osmotic minipump (model 2ML1). The pumps had been primed overnight to continuously administer spirolactone (Sigma-Aldrich, UK) at 20mg/kg/day (Macdonald *et al.*, 2000). Spirolactone was dissolved in 50% polyethylene glycol and the minipump was implanted as described (section 2.8.2).

2.9.3 Induction and SBP measurement

Animals were allowed 24h for recovery from the effect of surgery before SBP was measured daily over 3-4 days. The animals then received I3C by daily gavage for 3 days and SBP was measured daily over this period. At the end of induction renal function was measured as described (section 2.2 and 2.3).

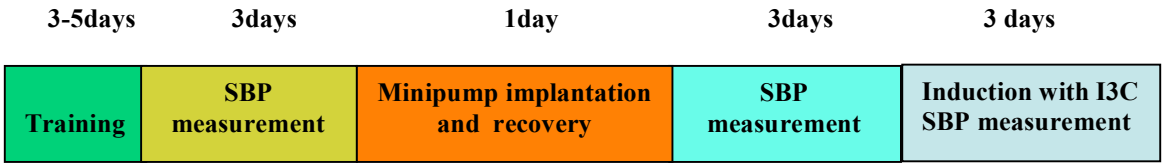


Figure 2.4: Experimental regime for MR antagonism study

2.10 Chronic inhibition of AT_1 receptor

A cohort of transgenic animals (n=6) was housed singly in normal cages and allowed free access to water and standard rat chow. Animals were trained for tail cuff SBP measurement as described above. Three consecutive readings of daily SBP were recorded as a baseline measurement. During this period, daily drinking rate was monitored by weighing the water bottle. The AT_1 receptor antagonist losartan (Fluka Analytical, Sigma-Aldrich, UK) was then added to the drinking water at a concentration designed to give a daily intake of 10mg/kg/day. The losartan-containing water was replenished daily. SBP was measured daily by tail cuff plethysmography as described (section 2.5) for 3 days of losartan treatment before the rats were induced with I3C for a further 3 days. At the end of induction renal function was measured as described (section 2.3 and 2.4).

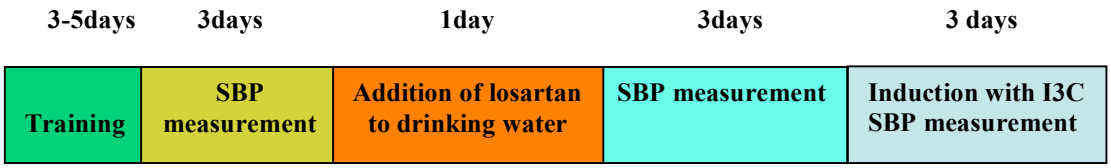


Figure 2.5: Experimental regime for AT_1 receptor antagonism study

2.11 Measurement of Glomerular Filtration Rate (GFR)

Glomerular filtration rate (GFR) was calculated by the clearance of FITC-inulin as detailed (Lorenz, 2002) using the following equation:

$$GFR = U_{Inu}V / P_{Inu}$$

where U_{Inu} = Inulin concentration in urine, P_{Inu} = Inulin concentration in Plasma and V = urine flow rate.

Urine samples were diluted at a 1:100 ratio in HEPES buffer (pH 7.4; Sigma-Aldrich, UK) as the fluorescence of FITC is pH-sensitive. Then 5µl of pre-diluted urine, plasma and standards (ranging from zero to 1mg/ml) were added with 195µl of HEPES to a black 96 well plate (Microfluor Solid Microplate, Nunc, Thermo Scientific). FITC-inulin concentration in urine and plasma were measured by fluorescence with excitation at 485nm and emission at 538nm by using a Wallac multilabel counter (model 1420; Perkin Elmer, Waltham, Massachusetts, USA).

2.12 Measurement of Renal Plasma Flow (RPF)

RPF was measured by the clearance of PAH and calculated using the following equation

$$\mathbf{RPF = U_{PAH}V / P_{PAH}}$$

where U_{PAH} = PAH concentration in urine, P_{PAH} = PAH concentration in plasma and V = urine flow rate.

The concentration of PAH was measured by using following method. Plasma samples were diluted with 3.2% trichloroacetic acid (TCA; Sigma-Aldrich, UK) at 1:20 dilution and vortexed briefly. A 10 minute equilibration at room temperature was followed by a second vortex mixing and another (5 minutes) incubation. Samples were then centrifuged (3 minutes at 16,000g). 500µl of supernatant was directly transferred to an eppendorf tube. Urine was diluted in 3.2% TCA (1:8000) and 500µl of the urine sample were taken. 25µl of each standard were taken in duplicate to eppendorf tubes ranging from zero to 1mg/ml and then diluted at a 1:20 ratio by adding 475µl of TCA. 100µl of 0.2N HCl solution and 50µl of 0.1% $NaNO_2$ (Sigma-Aldrich, UK) were added to all plasma, urine and standard samples which were vortexed and left for 2-3 minutes. After that 50µl of 0.5% ammonium sulfamate (Sigma-Aldrich, UK) was added and mixed by brief vortex. Finally 50µl of 0.1% N-(napthyl) ethylenediamide dihydrochloride (Sigma-Aldrich, UK) was added and mixed by vortex. The tubes were incubated at room temperature for between 15 min to 1hour to allow colour development. Colorimetric measurement was taken in spectrophotometer (Wallac 1420 Multilable HTS counter, model 1420; Perkin Elmer, Waltham, Massachusetts, USA) at 545nm. Concentrations were calculated from a standard curve.

2.13 Electrolyte and osmolarity measurement

2.13.1 Plasma and Urinary electrolyte measurement

Plasma sodium, potassium and lithium concentrations were measured by ISE electrolyte analyzer (Electrolyte analyzer 9180, Roche, UK) and urinary electrolytes

were analyzed by flame photometer (BWB Technologies, Essex, UK) following a 50 fold dilution in diluent (1% w/v Brij35, BWB Technologies, Essex, UK).

2.13.2 Faecal electrolyte measurement

Faeces collected during the metabolic cage study were heated at 100°C until constant weight was obtained. Dried faeces were then dissolved in 30ml of 0.7 M HNO_3 and homogenized with a homogenizer (Ultra Turrax, Model T20, Janke & Kunkel, Germany). After gentle shaking for the next 24h, samples were briefly spun and electrolytes were measured in the supernatant sample by flame photometer (BWB Technologies, Essex, UK).

2.13.3 Plasma and Urinary osmolarity measurement

Plasma and urine (20µl) osmolarity was measured through freezing point depression method by using a commercially available instrument (Vogel OM801, Germany).

2.14 Urinary Aldosterone measurement

Urinary aldosterone concentration was measured by ELISA analysis as described below (Al-Dujaili *et al*., 2009).

2.14.1 Assay reagents

Anti-aldosterone antibodies were raised in sheep (Al-Dujaili *et al*., 1981) and diluted in the ratio of 1:100,000 for use. HRP–Donkey-anti-sheep IgG enzyme conjugate was prepared in our laboratory following the Lightning-Link HRP technique (Innova

Biosciences, Cambridge) and used at the dilution of 1:2000 to develop the ELISA method. Aldosterone standard (Sigma–Aldrich, Poole, England) and aldosterone–BSA conjugate (Steraloids, USA) were purchased commercially.

2.14.2 Buffers

Assay buffer: 50 mmol/l phosphate buffered saline (PBS) pH 7.4 containing 0.1% bovine serum albumin (BSA, Sigma–Aldrich, UK).

Blocking buffer: 25 mmol/l PBS pH 7.4 containing 0.5% BSA.

Coating buffer: 25 mmol/l PBS pH 7.4.

Wash buffer: 15 mmol/l PBS pH 7.4 containing 0.05% Tween 20 (Sigma–Aldrich).

Substrate buffer: 200 mmol/l acetate/citrate buffer pH 4.2.

Stop solution: 1 mol/l sulphuric acid in distilled water.

Substrate solution: 11ml of substrate buffer+300ul substrate mix (10mg/ml Tetramethylbenzadine [Sigma–Aldrich, UK] in DMSO)+ 60ul H_2O_2 (Sigma–Aldrich, UK)

2.14.3 Urine extraction of aldosterone

Aldosterone was extracted from urine collected from the metabolic cage study described above. 0.5ml of urine was taken in a glass-stoppered tube and then aldosterone was extracted with 10 ml dichloromethane (Fisher Scientific, Leicester, UK) by vortex. Resultant extract was washed successively with 1 ml aliquots of 0.05 M NaOH, 0.05 M HCl and distilled water. The solvent was then dried at 40°C and the residue was reconstituted by adding 0.5 ml assay buffer.

2.14.4 Aldosterone ELISA Assays

96 well ELISA plates (Griener Bio-One, Germany) were coated with aldosterone–3-CMO-BSA conjugate (200 μl coating buffer containing 0.2 g conjugate/ml [Steraloids, USA]). The plates were then left overnight at 4°C covered with film. The coating solution was discarded and plates were washed three times with 250 μl wash buffer. Plates were then blocked by adding 200 μl blocking buffer and left at 37°C for 1h for incubation. Next the blocking buffer was discarded and plates were blotted and dried in air. Then 50 μl of urine extracts and aldosterone standards (range from 0–100 nM) were added to the wells followed by 100 μl of aldosterone antibody solution. Plates were then left for 2h at room temperature for incubation. At the end of the incubation the contents were discarded and the plate washed with buffer as before. Next 100 μl of HRP–anti-sheep enzyme conjugate was added to each well and the plates were covered and left for 1h at room temperature for incubation. Plates were emptied and washed as before and then after adding 100 μl substrate solution plates were left for 15 minutes in dark at room temperature. Finally, 50 μl of stop solution was added and absorbance was read at 450 nm in an MRX plate reader (Dynatech MRX, Dynatech. Laboratories, Billingshurst, UK).

2.14.5 Calculation of results

Aldosterone concentration in the sample was calculated by using the semi-log cubic spline software data reduction method built into the MRX ELISA reader (MRX EndPointSoftware ver. 1.33). After taking account of urine volume, values are then expressed as pmole/day ± SE.

2.15 Urinary albumin Measurement

Rat urine albumin measurements were determined using a commercial Microalbumin Kit (Olympus Diagnostics Ltd, Watford, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK). The immunoturbidimetric assay was standardised against purified albumin standards (Sigma Chemical Co. Poole, UK) with samples diluted in phosphate buffered saline as appropriate.

2.16 Protein Abundance measurements

2.16.1 Kidney homogenization

Initially during the optimization of Western blot analysis, two different lysis buffers were tried to extract the protein from whole kidney sample. The first buffer (buffer 1) was composed of 20mM HEPES (pH 7.4), 5mM EDTA, 5mM $Na_2P_4O_7$, 1% triton x-100, 1mM NaV, 10mM NaF, 1% protease inhibitor cocktail. The second lysis buffer was composed of 250mM sucrose (BDH, Poole, UK), 10mM triethanolamine (BDH, Poole, UK), 2% protease inhibitor cocktail (Pierce Protein Research Product, Thermo Scientific) and pH was adjusted to 7.6. As expected extraction was obtained with lysis buffer 2, this buffer was used to extract protein for the experiment using the following method. Stored kidneys were thawed at 4°C and then placed in ice-cold lysis buffer (10ml/gm kidney). Kidneys were homogenized by using a homogenization drill (T-25 digital, ULTRA –TURRAX, IKA, Werke GmbH & Co, Germany). Homogenization was carried out in a random order to avoid any bias resulting from protein degradation. After proper homogenization the tubes were left

on ice for 15 minutes as this was shown to give better extraction of protein. Homogenates were then centrifuged at 1000g for 5 minute at 4°C to separate debris. The supernatant was aliquoted and stored at -20°C. A sample was taken to determine the protein content by BCA protein assay.

2.16.2 Determination of protein content

Protein samples were diluted 100 times in deionised water. Bovine serum albumin (BSA) standards were prepared from the 2.0 mg/ml stock solution provided with the assay kit (The Thermo Scientific, Pierce BCA Protein Assay Kit). 40µl of each standard and sample were taken into a test tube and 800µl of the working reagent (50:1, reagent A:B, provided with assay kit) was added to each tube and mixed well. Tubes were incubated for 30 minutes at 60°C in a water bath and then cooled to room temperature. Now the spectrophotometer (Utrospec 2000, Pharmacia Biotech, Cambridge, UK) set to 562 nm, was zeroed on a cuvette filled only with water. Subsequently, the absorbance at 562 nm was measured for all the samples within 10 minutes. The average 562 nm absorbance measurement of the blank standard (water only) replicates was subtracted from the 562 nm absorbance measurement of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected 562 nm measurements for each BSA standard against concentration (µg/ml). The standard curve was used to determine the protein concentration of each unknown sample. Standards and unknowns were measured in duplicate.

2.16.3 SDS-polyacrylamide gel electrophoresis

2.16.3.1 Gel Preparation

A Bio-Rad mini cell system (Bio-Rad Laboratories, Hercules, CA, USA) was used to prepare gels for protein electrophoresis. Glass plates and spacer plates were rinsed with 70% ethanol and left to dry before being assembled in the gel casting frame. A sufficient amount of 10% polyacrylamide running gel was prepared (0.375M Tris-Cl [1.5M Tris-Cl pH 8.8; Sigma-Aldrich, UK], 0.1% SDS [sodium dodecyl sulphate; Sigma-Aldrich, UK], 10% acrylamide [Bio-Rad Laboratories, Hercules, CA, USA], TEMED [0.05% tetramethylethylenediamine; Sigma-Aldrich, UK]) and freshly prepared APS (0.067% ammonium peroxy disulphate; Sigma-Aldrich, UK) were added immediately before use to cause gel polymerization. The solution was then poured between the glass plates and water saturated butanol was added to create a flat transition front. After this, the running gel was left 15-30 minutes to set at room temperature. In the meantime, a 4% stacking gel was prepared (0.12 M Tris-Cl [1.5M Tris-Cl P^H 6.8], 0.1% SDS, 4% Acrylamide, 0.05% APS and .067% TEMED).

Once the above mentioned running gel was set, the butanol was poured off followed by a rinse with deionized water. The stacking gel (~5 ml) was then poured onto the running gel and a comb was inserted immediately. At this point the stacking gel was left for 15 minutes to set at room temperature. The gel was then fitted with gel tank (Mini PROTEAN 3 Cell, Bio-Rad Laboratories, Hercules, CA, USA) and immersed in buffer. The comb was removed and the wells were flushed out thoroughly with running buffer (0.025M Tris, 0.1% SDS, 0.192M glycine in deionised water).

2.16.3.2 Sample preparation and loading

Samples were taken out from -20°C and thawed on ice. An appropriate amount of protein sample containing 50µg of total protein was taken in a fresh eppendorf tube. Now 4µl of 5X laemmli buffer (for 1X, 2% SDS, 50mM Tris, 0.01% bromophenol blue, 10% glycerol) and 1µl of 2M DTT was added and then deionised water was added to make a final volume of 20µl. Samples were mixed and heated at 60°C for 10 minutes. The wells were then loaded with 20µl of sample by using gel loader tips (Eppendorf, UK). 5µl of rainbow marker (Fermentas Life Science) and 5µl Cruz marker (Santa Cruz Biotechnology) were also loaded in two separate wells randomly to identify the molecular weight of bands. The gels were run at a constant voltage of 150 V for 60 minutes. Separation of coloured protein markers confirmed electrophoresis was a success.

2.16.3.3 Membrane Transfer

PVDF membrane (Amersham Biosciences) and blotting paper (Bio-Rad Laboratories, Hercules, CA, USA) of appropriate size (cut to the dimensions of the gel, approximately 6x9 cm) were prepared in advance. PVDV membrane was submerged in methanol and blotting paper in transfer buffer. After 5/10 minutes the membrane was taken out from methanol and submersed in transfer buffer. Complete wetting of the membrane is important to ensure proper binding of proteins. Once the proteins were separated the glass plates were taken out carefully and immersed into transfer buffer. The gel was then carefully taken out from the glass plates and the stacking gel was stripped off from the running gel. This equilibration of gel in transfer buffer helps facilitate the removal of electrophoresis buffer salts and

detergents. Moreover equilibration allows the gel to adjust to its final size prior to electrophoretic transfer. Now one of the pre-soaked sheets of blotting paper was placed onto the platinum anode. Then all air bubbles were excluded by rolling a pipette over the surface of the blotting paper. Subsequently, the pre-wetted PVDF membrane was placed on top of the wetted blotting paper and the air bubbles rolled out as before. Now the equilibrated gel was placed on top of the PVDF membrane, aligning the stack as perfectly as possible. Finally another piece of pre-soaked blotting paper was placed on top of the gel. Air bubbles were removed from between the gel and filter paper. Once this gel/PVDF stack was complete it was placed onto a semi-dry transfer unit (Trans-Blot SD, Bio-Rad Laboratories, Hercules, CA, USA) at 30V for 30 minutes.

2.16.3.4 Immunobloting

Once the transfer was completed the membrane was blocked in 20 ml of either 5% dried skimmed milk (Marvel, Premier Brands UK Limited, Spalding, Lincolnshire) or 3% BSA (BDH, Poole, UK) in TBS Tween (20mM Tris-Cl [0.2 M Tris-Cl, pH 7.6], 137mM Sodium Chloride, 0.1% Tween 20 [Polyoxyethylenesorbitan monolaurate, BDH, Poole, UK]) for 1 hour at room temperature on a platform shaker (Stuart Scientific, Staffordshire, UK) at the speed of 40unit. At the end of blocking, 5ml of blocking solution was taken in a universal tube and an appropriate amount of primary antibody was added (Primary antibody dilutions are given below). Now the membrane was rolled into a 50 ml Falcon tube and left overnight at 4°C. The

remaining blocking solution was kept at 4°C to use during probing with secondary antibody.

The overnight-probed membrane was washed 3 times (5 minutes each) in TBS Tween using platform shaker at the speed of 60 rev/min. Then peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was added with blocking solution (1:10000 dilution) in a Falcon tube. Then the membrane was rolled into the tube carefully and incubated for 1 hour at room temperature on an orbital shaker (Stuart Scientific, Staffordshire, UK). After the incubation, membrane was washed 3 times as described earlier. The membrane was then incubated with an ECL-plus Western blotting detection system (Thermo Scientific, IL, USA) following the manufacturer's instructions. Excess ECL substrate from the membrane was removed by putting the membrane over a piece of folded tissue paper and briefly touching the edge of the membrane to the paper. Then the membrane was placed down on thin plastic, the bubbles were removed and the membrane was wrapped completely. The membrane was taped inside a film cassette and exposed to a photographic film (BioMax XAR Film, Kodak, Sigma-Aldrich, UK) for an adequate exposure time. The film was developed and immunoreactivity was quantified by densitometry by using ImageJ (Abramoff *et al*., 2004).

2.16.4 Antibody source and dilutions

Initially polyclonal rabbit anti-rat NCC was purchased from Alpha Diagnostics (San Antonio, TX, USA), but due to very strong background noise, polyclonal anti-NCC from Chemicon (Millipore, MA, USA) was used. For α-ENaC, polyclonal antibody raised against rat α-ENaC produced in rabbit was purchased from Upstate (NY,

USA). Polyclonal Sgk-1 rabit anti-Sgk-1 was purchased from Upstate (NY, USA). For nedd4, antibody raised in rabbit was purchased from Upstate (NY, USA). NKCC1 specific antibodies N1 and T4 (also binds with NKCC2) were a gift from Dr. Peter W Flatman. Goat anti rabbit Immunoglobulins conjugated with horseradish peroxidase purchased from Promega, UK was used as secondary antibody. Optimum dilutions for primary and secondary antibody are shown in the below table.

Protein Name	Primary antibody dilution	Secondary antibody dilution
T4	1:10000	1:10,000
N1	1:5000	1:10,000
NCC	1:2500	1:10,000
α-ENaC	1:2000	1:10,000
Sgk-1	1:5000	1:10,000
Nedd4-1	1:5000	1:10,000
GAPDH	1:5000	1:10,000

2.16.5 Assessment of equal loading

To assess equal loading of protein, GAPDH primary antibody (R&D Systems, UK) was used. The Western blot was incubated with GAPDH antibody (1:5000) for 1 hour at room temperature on an orbital shaker. Following washing, the blot was probed with goat anti rabbit secondary antibody for 1 hour at room temperature on an orbital shaker. Then membrane was developed following the method described above. A protein of 36 KDa size appeared and equal loading was confirmed by densitometric analysis (Figure 2.6).

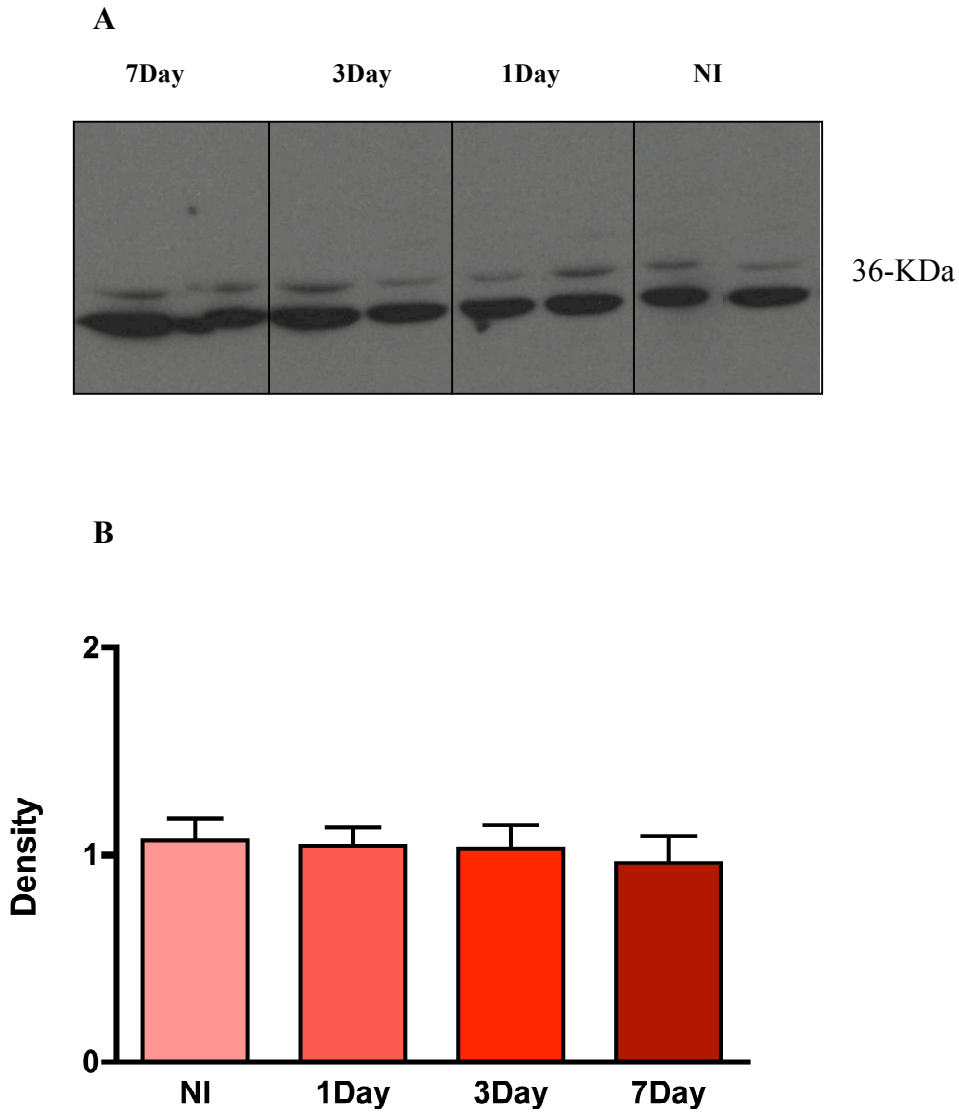


Figure 2-6: Representative blot for whole kidney GAPDH protein abundance in Non-Induced (NI, n=6), 1-day Induced (1Day, n=6), 3-day Induced (3Day, n=6) and 7-day Induced (7Day, n=6). Western blot analysis identified a protein band at approximately 36 kDa (A). Summary of band density of 4 groups of rats by densitometry quantification is shown in (B). All groups showed similar density levels.

2.17 Statistical analysis

Statistical comparisons were made using an unpaired t-test, one-way ANOVA or two-way ANOVA as appropriate (Tukey posthoc test) using GraphPad Prism software (Graphpad software, San Diego California, USA). Statistical significance was defined as $p<0.05$ and all data are expressed in mean±SE.

Chapter 3

In vivo renal function in Cyp1a1-mRen2.F transgenic rat

3.1 Renal function in the anaesthetized rat: time control study

Time-controlled experiments were conducted to evaluate the stability of the rat's renal function after surgical preparation for renal clearance study. In the present study a group of six Fischer rats (Harlan, UK) were prepared surgically for renal clearance. Following one hour equilibration, three urine collections, each of which was forty minutes, were performed. Renal haemodynamics blood pressure, urine flow rate and urinary sodium excretion were measured throughout (a detailed protocol is described in figure 2.1A in the methods section). Data obtained in this study were used to confirm that any changes observed in the following studies could be attributed to the administration of drug rather than being an effect of time or vehicle.

3.1.1 Blood pressure and renal haemodynamics

Figure 3-1 shows the mean arterial blood pressure (MABP), glomerular filtration rate (GFR) and renal plasma flow (RPF) over three collection periods. The rats had a stable blood pressure throughout the experiment, with no significant difference between the MABPs recorded in each of the collection periods (Figure 3-1A). Similarly there were no significant changes in GFR and RPF throughout the experiment (Figure 3-1B and 3-1C respectively).

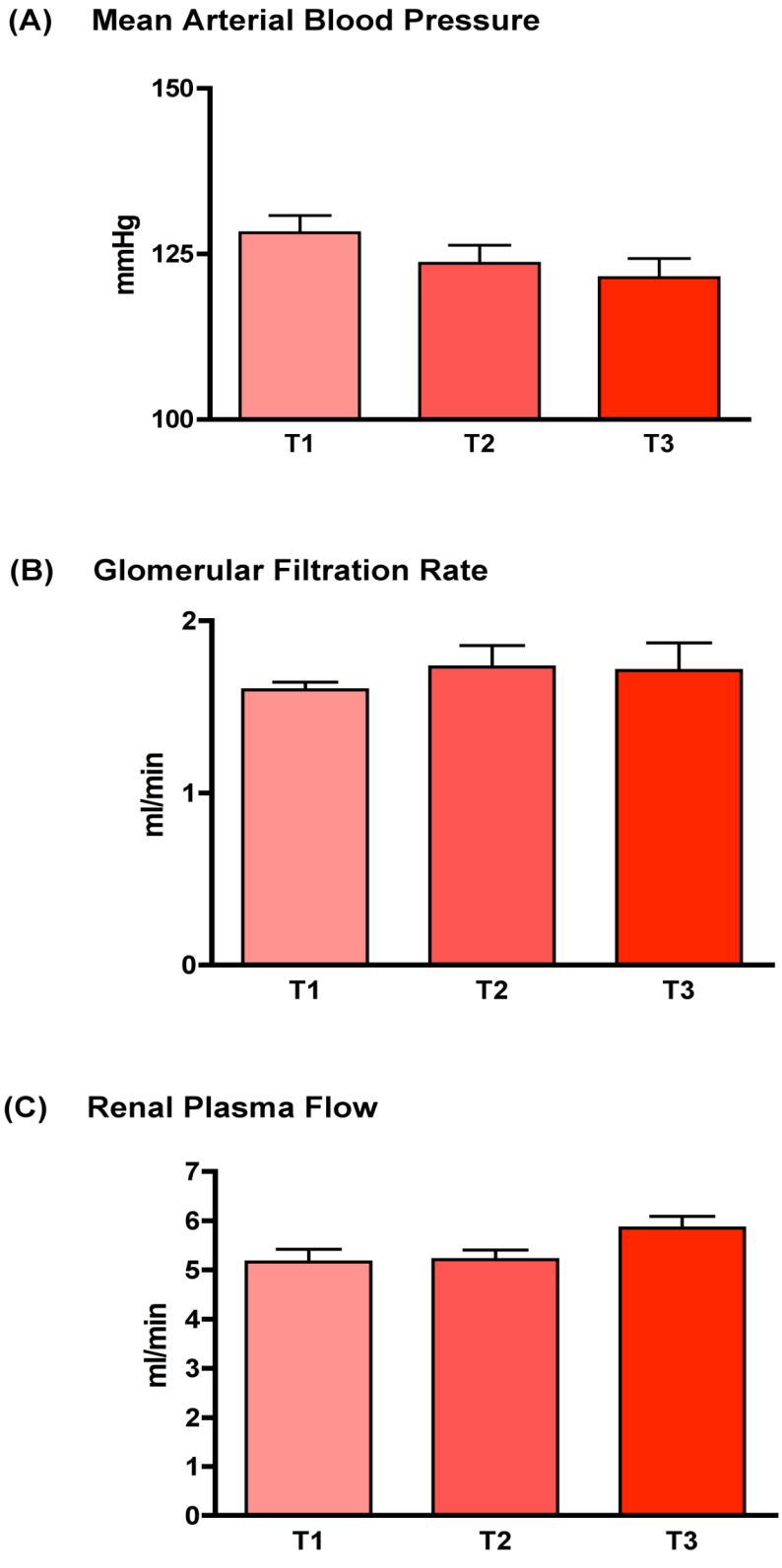


Figure 3-1: (A) MABP (B) GFR (C) RPF in Fischer non-induced (N=6) rats during three consecutive periods (T1, T2 and T3) of urine collection. Data are mean±SE and statistical comparison was made by one way ANOVA. There were no significant differences.

3.1.2 Urine flow and urinary sodium excretion

Figure 3-2 shows urine flow rate and urinary excretion of sodium. Both urine flow rate and urinary sodium excretion were stable throughout the experiment, with no significant differences measured in the three collection periods.

These data indicate that the surgical preparation and infusion protocol have no significant effect on the rat's renal haemodynamics, blood pressure, urine flow rate and urinary sodium excretion. Consequently, protocols of this duration can be used to evaluate the effect of pharmacological intervention on renal function in future studies.

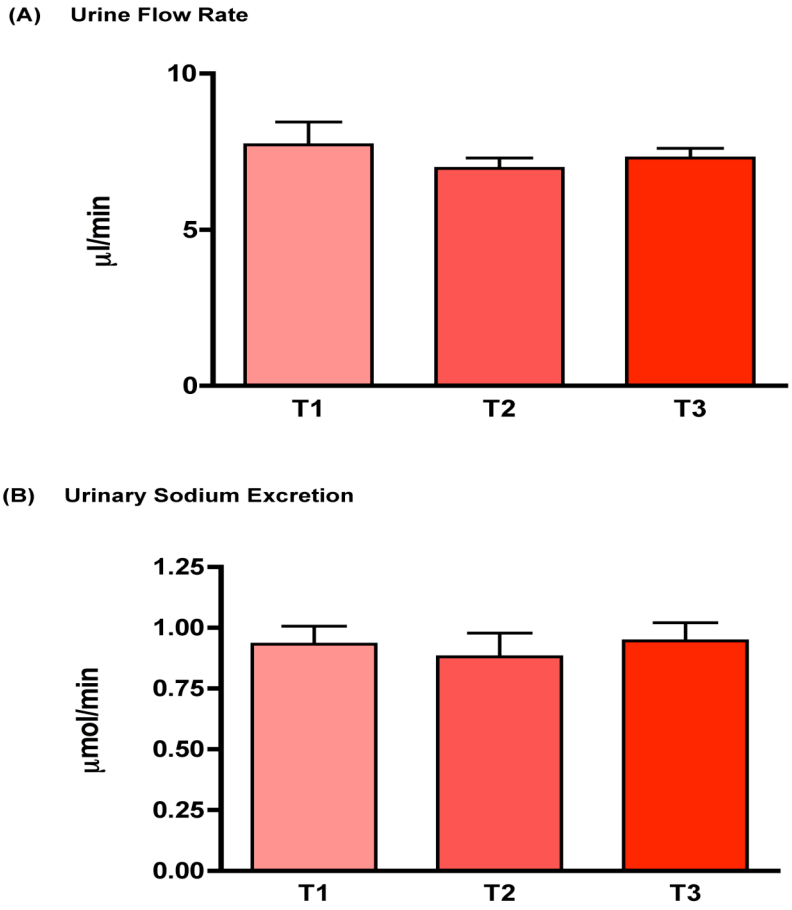


Figure 3-2: (A) Urine Flow Rate (B) Urinary Sodium Exretion in Fischer non-induced (N=6) rats during three consecutive periods (T1, T2 and T3) of urine collection. Data are mean±SE and statistical comparison was made by one way ANOVA. There were no significant differences.

3.2 Selection of appropriate genetic control group

For control purposes, two groups of rats were used. The first group was non-transgenic Fischer rats (F344 from Harlan, UK) and the second group was Cyp1a1-mRen2 transgenic rats on a Fischer 344 background. These rats received vehicle (vegetable oil) by gastric gavage for seven days before the experiment started.

3.2.1 Blood pressure and renal haemodynamics

MABP, GFR and RPF for both groups of rats are summarised in Figure 3-3. There was no difference in MABP between the two control groups. Both GFR and RPF were also comparable between groups.

3.2.2 Urine flow and urinary sodium excretion

Figure 3-4 shows the urinary sodium excretion and urine flow rate for Cyp1a1-mRen2.F transgenic and non-transgenic control rats. No difference in urinary sodium excretion and urine flow rate were observed between the Cyp1a1-mRen2.F transgenic and non- transgenic control. Moreover no difference in fractional excretion of sodium indicates that tubular sodium sodium reabsorption is also comparable between two groups. Plasma electrolytes, plasma osmolarity and urine osmolarity data are summarised in table 3.1.

	Non-transgenic rats (n=8)	Transgenic rats mRen2.F (n=8)
HCT	46.93±0.97	46.5±0.98
P_{Na} (mmol/L)	135.7±1.41	135.9±0.51
P_K (mmol/L)	4.54±0.18	4.43±0.48
P_{Li} (mmol/L)	1.29±0.05	1.24±0.04
P_{OSM} (mOSM/Kg)	286±2.73	289±2.83
U_{OSM} (mOSM/Kg)	1637±81.17	1697±67.32

Table 3.1: Plasma data from both transgenic and non-transgenic control groups. P_{Na}, P_K, and P_{Li} are the plasma concentration of sodium, potassium and lithium respectively. P_{OSM} and U_{OSM} are the plasma osmolarity and urine osmolarity respectively. Values are Mean±SE and statistical comparison was made by unpaired t-test. There were no significant differences.

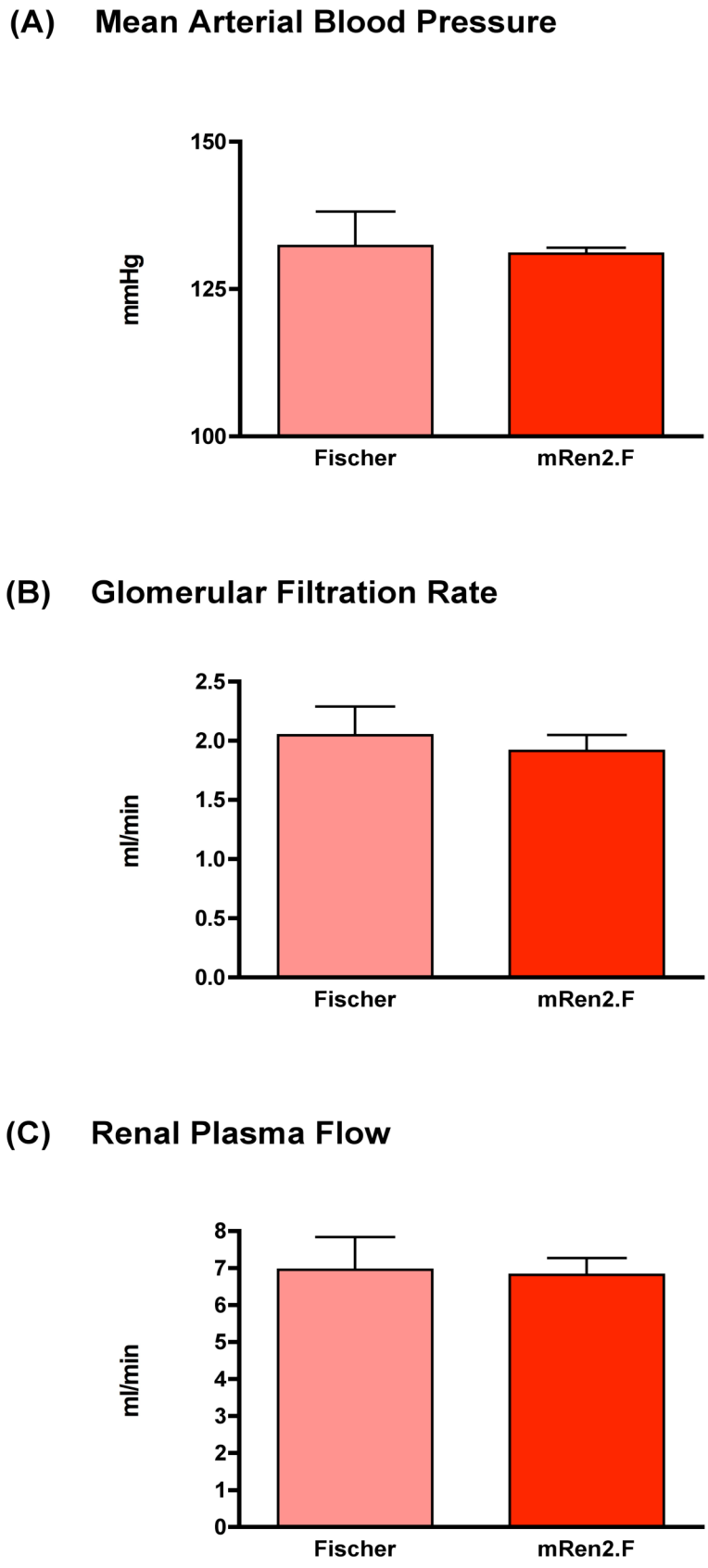


Figure 3-3: (A) MABP (B) GFR (C) RPF in non-transgenic Fischer control rats (n=8) and Cyp1a1-mRen2.F transgenic control rats (mRen2F, n=8). Data are mean±SE and statistical comparison was made by unpaired t test. There were no significant differences between two groups.

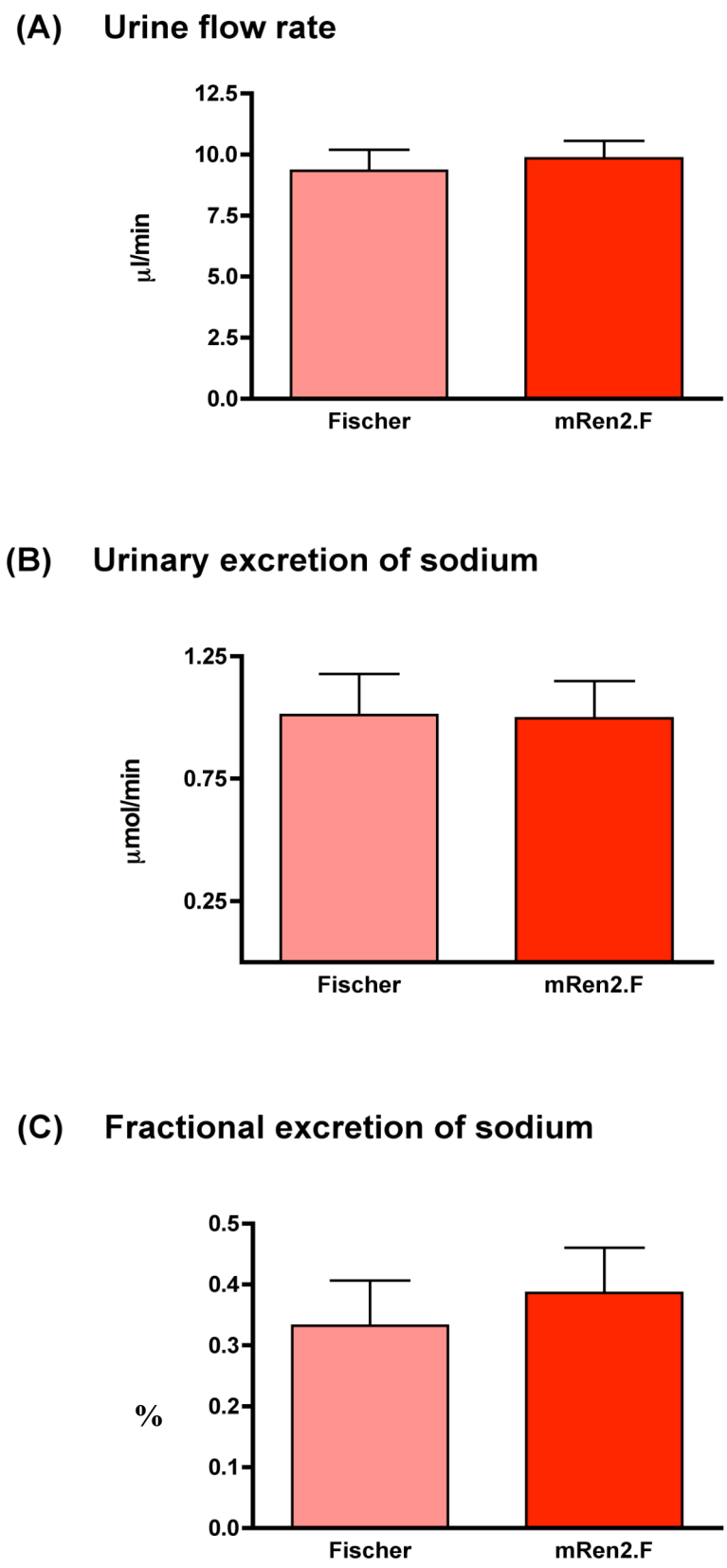


Figure 3-4: (A) Urine flow rate (B) Urinary excretion of sodium (C) Fractional excretion of sodium in non transgenic Fischer control rats (n=8) and Cyp1a1-mRen2.F transgenic control rats (mRen2.F, n=8). Data are shown as mean±SE and statistical comparison was made by unpaired t test. There were no statistical significant differences between two groups.

3.3 Effect of Cyp1a1-mRen2 transgene induction on renal function

3.3.1 Development of hypertension

I3C (100mg/kg of body weight) was administrated to Cyp1a1-mRen2.F transgenic rats by daily gavage for 1, 3 and 7 days. Daily measurement of mean blood pressure by tail cuff plethysmography for rats induced for 7 days is shown in figure 3-5A. Blood pressure was found to be elevated from the first day of induction and further elevation was observed as the experiment progressed. The development of hypertension was accompanied by a progressive loss of body weight (Figure 3-5B). Body weight was decreased by day three of induction and continued to decrease for the rest of the induction period. After 7 days of induction rats lost 10% of body weight and also showed symptoms associated with malignant hypertension in rodents. Rats were hunched, inactive and had piloerection. These findings are consistent with the previous data using this dose of I3C (Liu *et al*., 2009). At the end of the period of induction rats were anaesthetized for MABP measurement and analysis of renal function. Direct measurement of MABP by cannulation identified that induction of the transgene caused a graded elevation of blood pressure (Figure 3-6A).

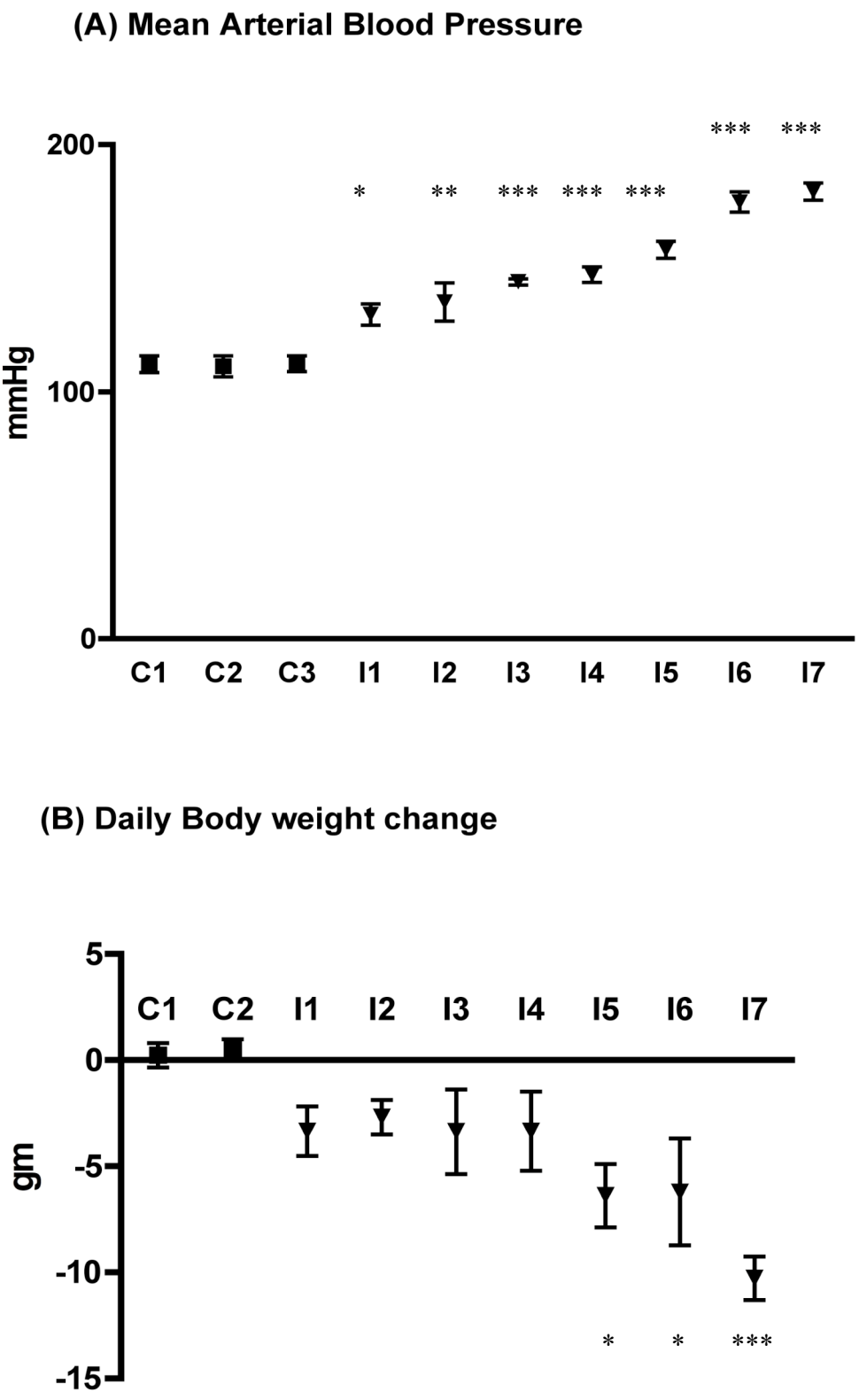


Figure 3-5: (A) Daily measurement of Mean Arterial Blood Pressure by tail cuff plethysmography (N=6) and (B) Daily body weight change. C1, C2 and C3 represent the control period without any induction. I1 to I7 represent the induction from day 1 to 7. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs mean of control period readings.

3.3.2 Renal haemodynamics

Figure 3-6B shows GFR in the four groups of animals. On average, GFR was higher in the 1-day induced group compared to the non-induced group but this difference was not statistically significant. However, after 3 days of induction there was a significant increase in GFR. This effect was transient; rats having 7 days of induction had a GFR comparable to non-induced controls.

Figure 3-6C shows RPF in the four groups of animals. In general, RBF mirrored the patterns of GFR, having a tendency to increase in groups induced for 1 day and 3 days. One-way ANOVA identified an overall significant difference but there was no significant difference between any induced groups compared to the non-induced group by post hoc analysis. Thus overall GFR and RPF remained unchanged despite the markedly elevated MABP followed by induction.

There was an overall statistical significant difference in filtration fraction found by one-way ANOVA but post hoc analysis did not show statistical significance between any induced group compared to the non-induced group (Figure 3-7A). Since RPF was normal even in the face of a markedly elevated blood pressure, these data are suggestive of a marked increase in RVR. Indeed no change in renal vascular resistance was observed in 1-day and 3-day induced groups but a significant increase was found after 7 days of induction (Figure 3-7B).

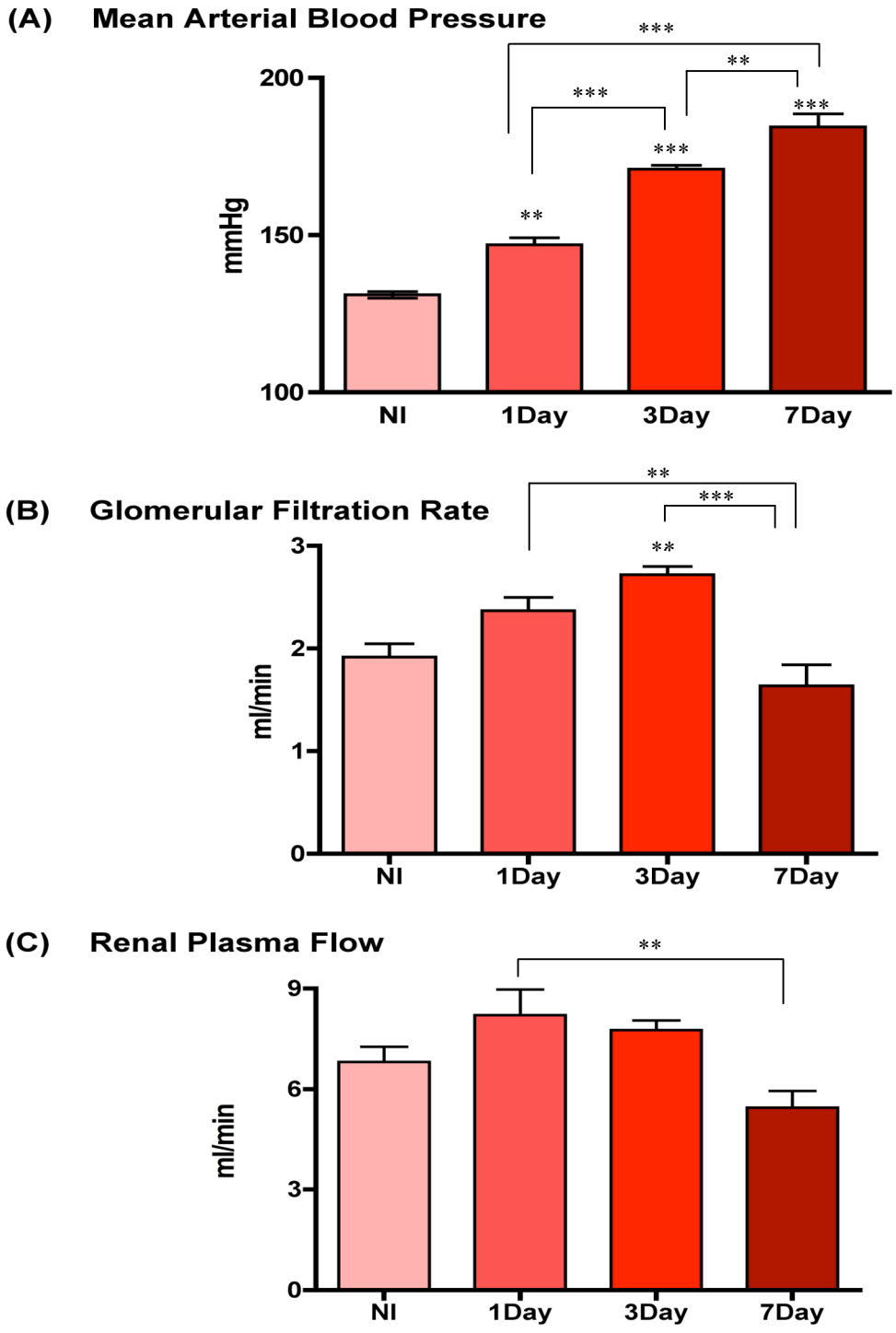


Figure 3-6: (A) MABP (B) GFR (C) RPF in non-induced (NI, n=8), 1-day induced (1day, n=9), 3-day induced (3day, n=9) and 7-day induced (7day, n=8) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. **=p<0.01 and ***=p<0.001 vs NI group.

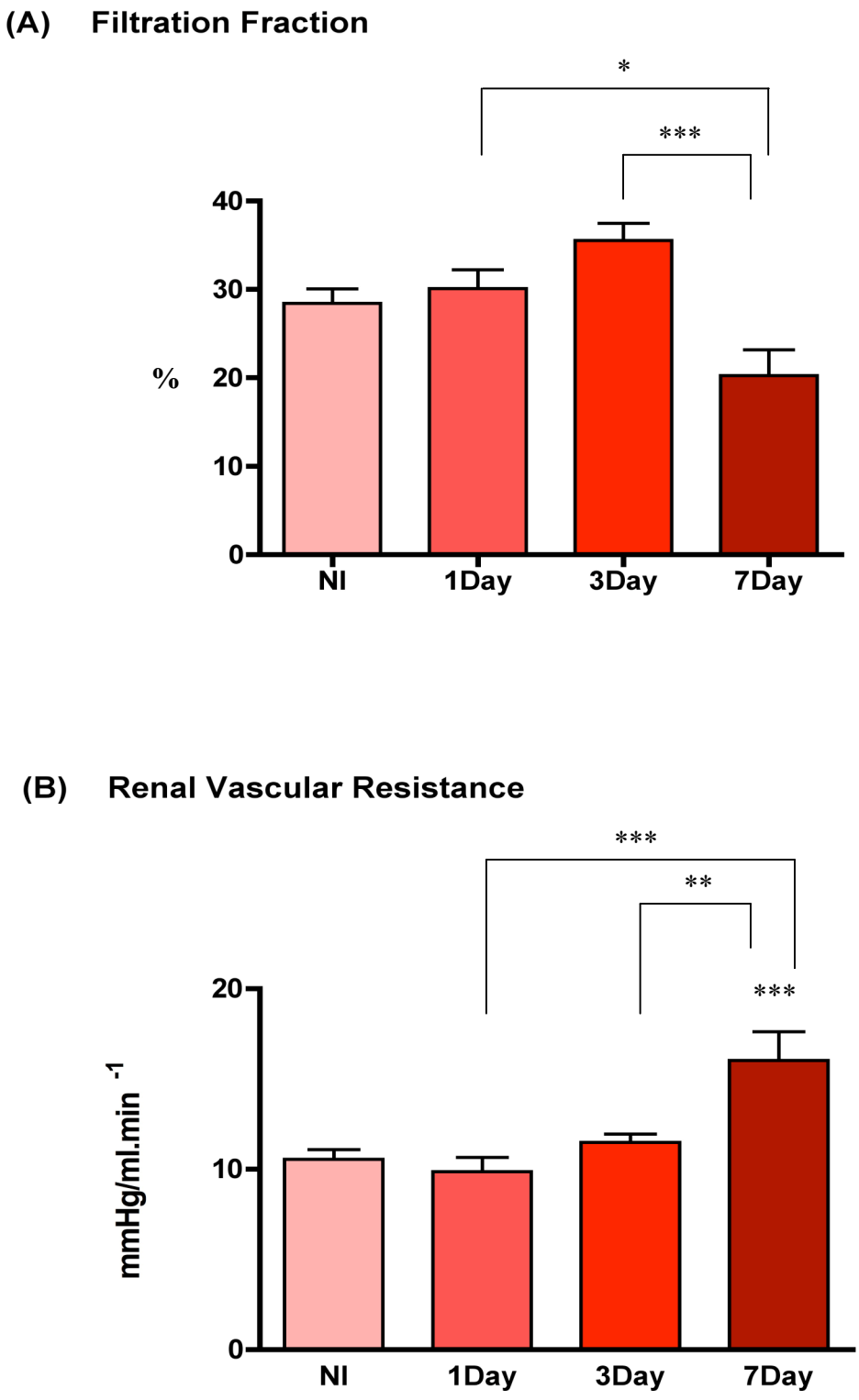


Figure 3-7: (A) Filtration Fraction (B) Renal Vascular Resistance in non-induced (NI, n=8), 1-day induced (1day, n=9), 3-day induced (3day, n=9) and 7-day induced (7day, n=8) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs NI group.

3.3.3 Renal handling of sodium

Absolute sodium excretion (E_{Na}) and fractional excretion of sodium (FE_{Na}) are shown in figure 3-8. Excretion of sodium was significantly lower in the 1-day and 3-day induced group compared to the non-induced group. A further incremental drop in sodium excretion was found in the 7-day induced group. Similar differences were also observed for fractional excretion of sodium. Since FE_{Na} normalizes E_{Na} for differences in plasma sodium concentration and GFR, these data reflect changes in tubular sodium reabsorption.

3.3.4 Renal handling of potassium

Figure 3-9 shows urinary potassium excretion and fractional excretion of potassium. Potassium excretion was comparable across all groups with the exception of a marked decrease in the 7-day induced group. No significant difference in fractional excretion of potassium (Figure 3-9B) indicates that the decrease in potassium excretion after 7 days of induction was not due to increased tubular reabsorption.

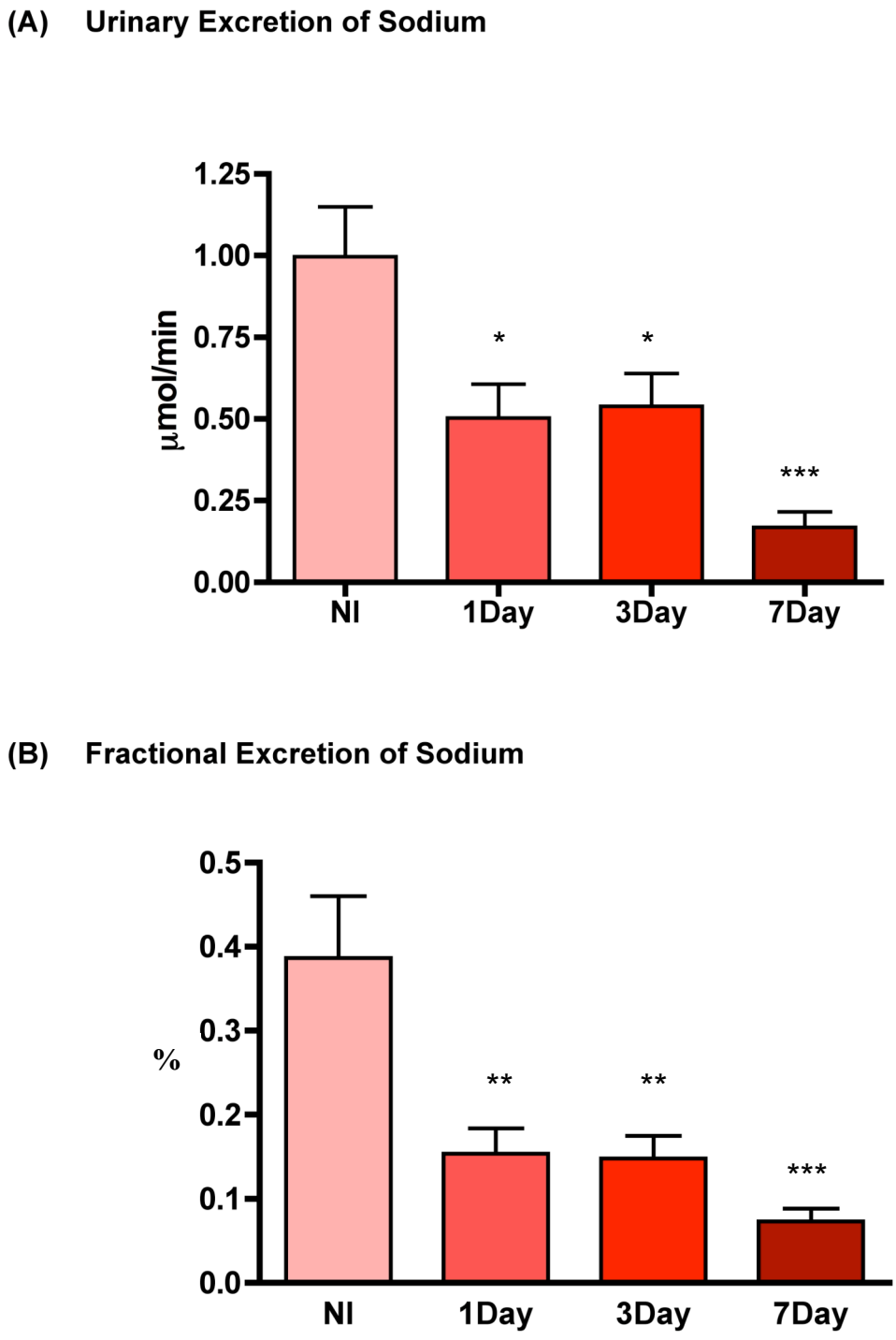
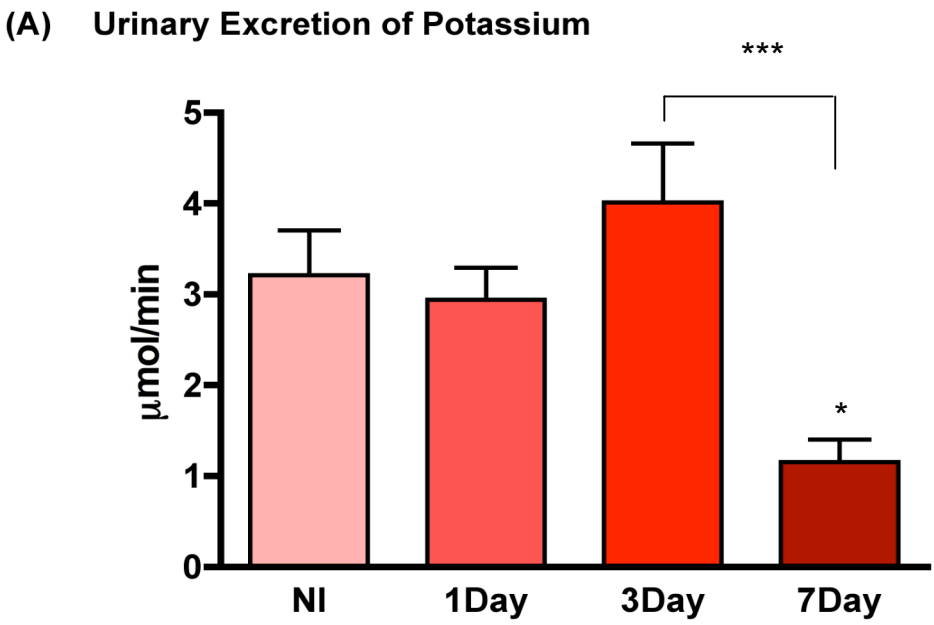


Figure 3-8: (A) Urinary Excretion of Sodium (B) Fractional Excretion of Sodium in non-induced (NI, n=8), 1-day induced (1day, n=9), 3-day induced (3day, n=9) and 7-day induced (7day, n=8) groups. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs NI group.



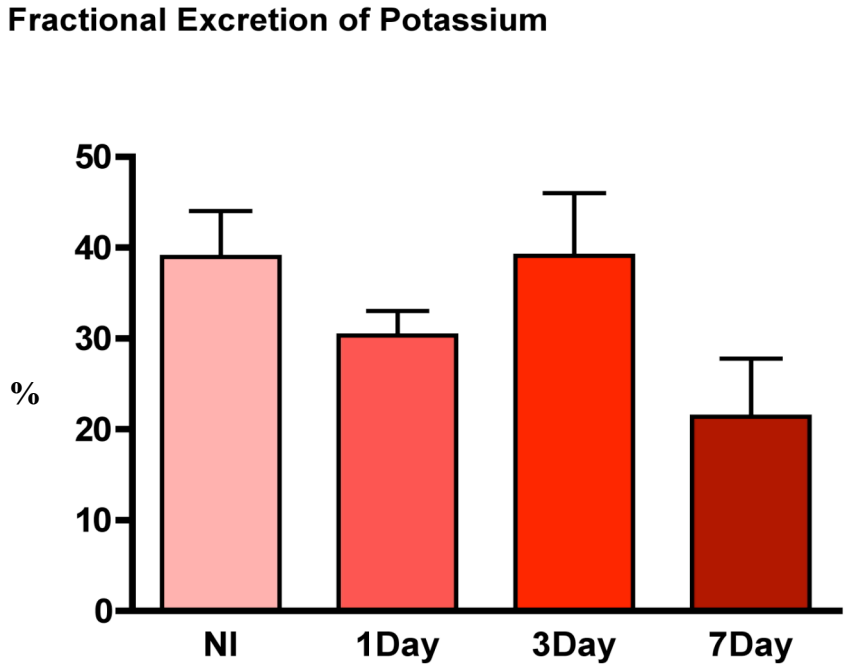


Figure 3-9: (A) Urinary Excretion of Potassium (B) Fractional Excretion of Potassium non-induced (NI, n=8), 1-day induced (1day, n=9), 3-day induced (3day, n=9) and 7-day induced (7day, n=8) groups. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and ***=$p<0.001$ vs NI group.

3.3.5 Renal handling of water

Osmolar excretion and free water clearance are summarised in Figure 3-10. Osmolar excretion remained stable in both 1-day and 3-day induced groups. But a significant decrease in osmolar excretion was observed after 7 days of induction. Free water clearance mirrored the osmolar excretion and a significant change in free water clearance was only observed after 7 days of induction.

Table 3.2: Renal data and plasma electrolytes in Cyp1a1-Ren2 transgenic rats which had induced hypertension for 1, 3 or 7 days. The control group received no induction. Data are mean±SE and "n" is given in parentheses. Comparisons were made using one-way ANOVA (P value shown), with Tukey's post hoc analysis: *=P<0.05, **=P<0.01. FE_{Li} indicates the fractional excretion of lithium; V is urine flow rate; C_{Na}/C_{Li} is the ratio of the clearances of sodium and lithium; P_{Na} and P_K are the plasma concentration of sodium and potassium; Hct is hematocrit;.

	Control (8)	1-day (9)	3-day (9)	7-day (9)	P
V (µl/min)	9.8±0.7	13.6±3.5	13.2±1.0	9.3±2.0	NS
FE_{Li} (%)	20.1±3.4	40.9±6.2**	38.8±4.9**	7.9±1.8*	<0.01
C_{Na}/C_{Li}	8.4±1.7	3.4±1.2*	3.9±1.4	2.8±1.0*	<0.05
Hct (%)	46.5±1.0	48.3±0.5	48.7±1.0	52.0±1.1**	<0.01
P_{Na} (mmol/l)	135.9±0.4	134.3±0.7	134.0±0.6	130.3±1.7*	<0.01
P_K (mmol/l)	4.23±0.06	4.10±0.12	4.03±0.11	3.87±0.09	NS

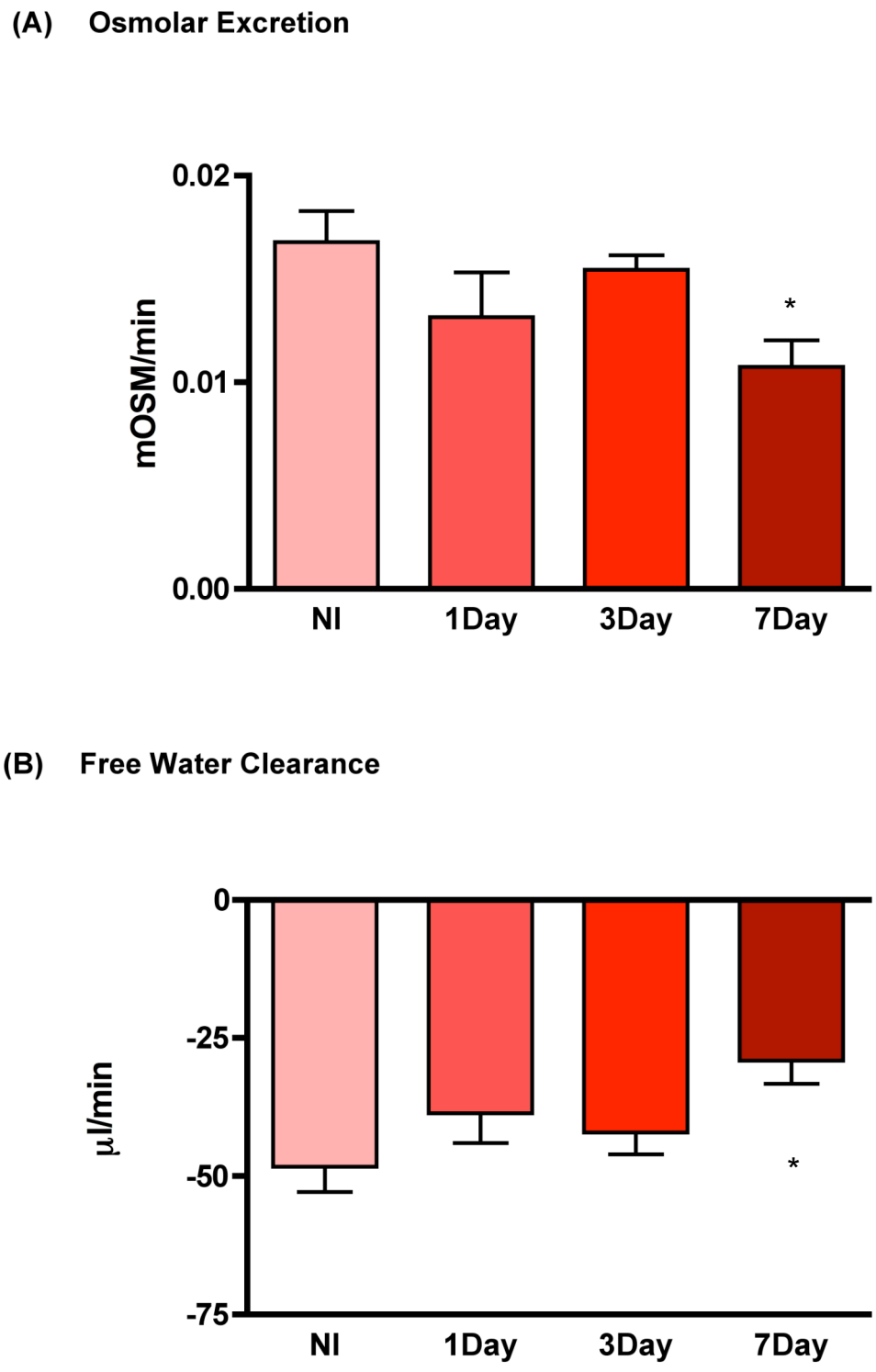


Figure 3-10: (A) Osmolar Excretion and (B) Free water Clearance in non-induced (NI, n=8), 1-day induced (1day, n=9), 3-day induced (3day, n=9) and 7-day induced (7day, n=8) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ vs NI group.

3.4 Daily sodium and water balance during transgene induction.

In order to investigate the effect of induction on daily sodium and water balance, a metabolic cage study was performed. In this study, following a three-day acclimatization period, control measurements were made over a further three days. Subsequently rats were induced by daily gastric gavage of I3C for seven consecutive days.

3.4.1 Daily Food and water consumption

Daily food and water intake are summarised in figure 3-11. Daily food intake measurement identified a trend of decreased appetite for food upon induction. Food intake fell from the beginning of induction and it continued to fall up to the end of induction. A significant decrease in food intake was observed on day three of induction and it continued to decrease during the remaining period of induction. This decrease in food intake was accompanied with a progressive loss of body weight throughout the induction period. Figure 3-11C shows the daily measurement of body weight for 3 days of control period without any induction and during 7 days of induction. By the end of 7 days of induction the rats had lost approximately 10% of baseline body weight.

Water intake remained comparable with the control period for the first three days of induction. From the forth day of induction water intake progressively increased with a maximum intake recorded (nearly doubled) at day six of induction.

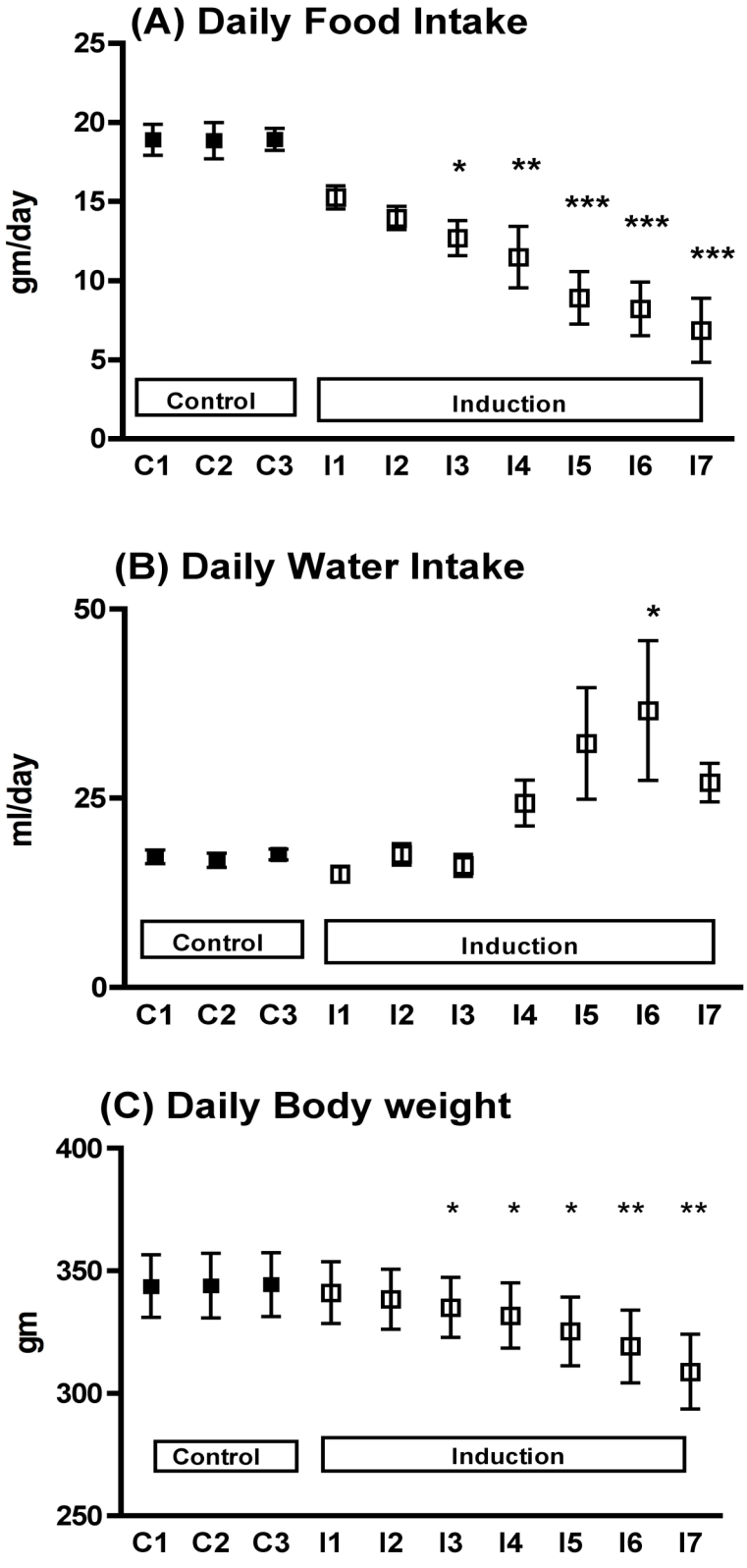


Figure 3-11: Daily measurement of (A) Food Intake, (B) Water Intake and (C) Body weight during control period and transgene induction. C1, C2 and C3 represent the control period without any induction. I1 toI7 represent the induction from day1 to 7. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs mean of control period readings.

3.4.2 Daily urine flow

Figure 3-12 shows the daily urine flow remained fairly steady up to the second day of induction during the metabolic cage study. In parallel with water intake, a trend of increased urinary flow was observed after the third day of induction. A significant increase was only found after the sixth day of induction. However no significant change in water balance was observed during any period of the study (Figure 3-12B). This suggests although there was an increased turnover of water, their balance was unchanged overall.

3.4.3 Daily urinary excretion of sodium

Daily sodium intake, urinary excretion of sodium and sodium balance are summarised in figure 3-13. In previous experiments it was shown that food consumption was reduced due to transgene induction. Consequently a reduction in sodium intake was also found following induction. This decrease in sodium intake was found to be significant only after day 3 of induction and decreased further over the remaining period of induction. Although there was a tendency towards an increase in urine flow, daily urinary excretion of sodium was found to be reduced after induction. This reduction in urinary sodium excretion was found to be statistically significant after 3 days of induction. However animals had a positive sodium balance throughout the experiment and no change in sodium balance was observed after transgene induction.

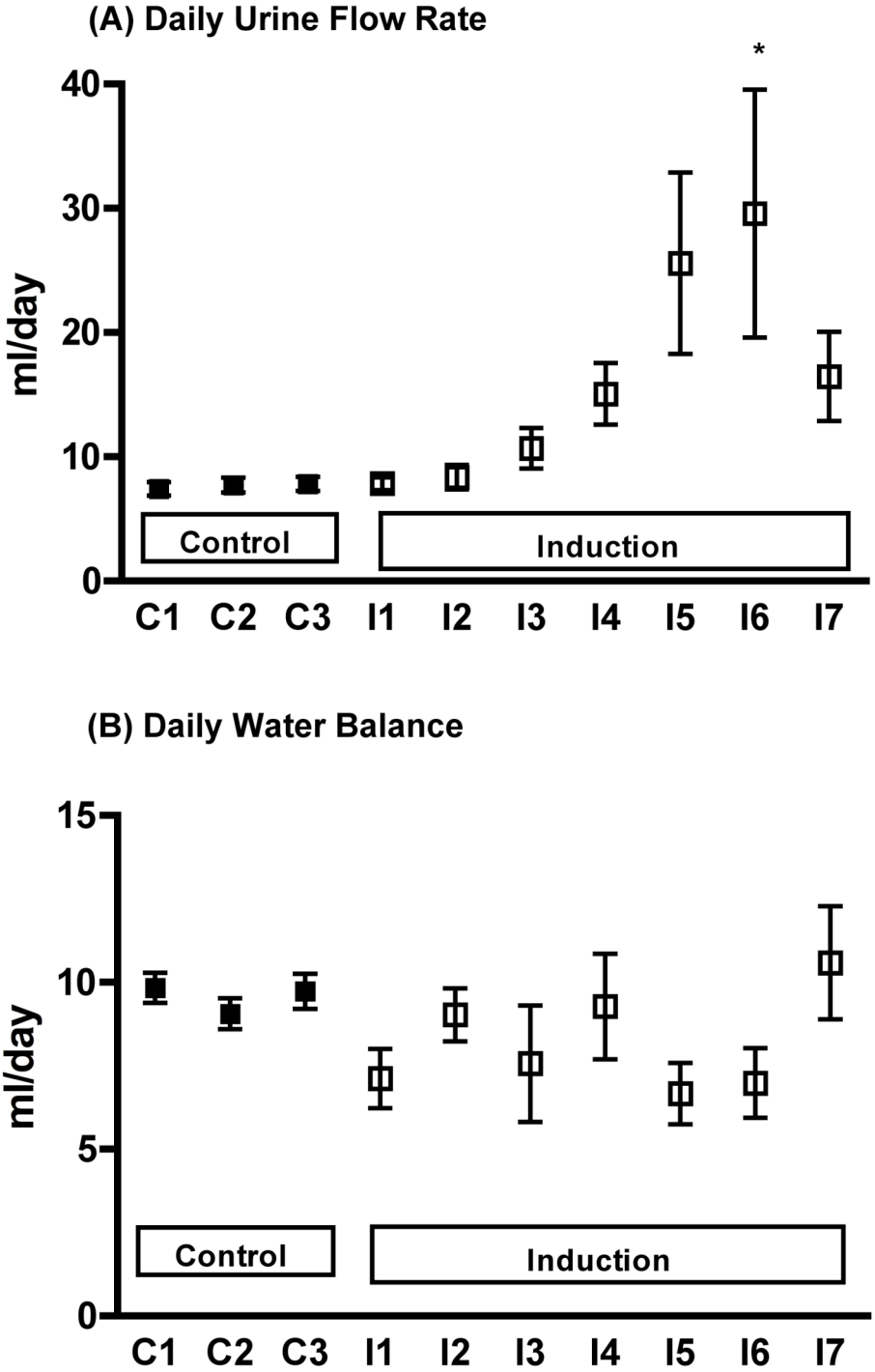


Figure 3-12: Daily measurement of (A) Urine Flow Rate and (B) Water Balance during control period and transgene induction. C1, C2 and C3 represent the control period without any induction. I1 to I7 represent the induction from day 1 to 7. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. *=$p<0.05$ vs mean of control period readings.

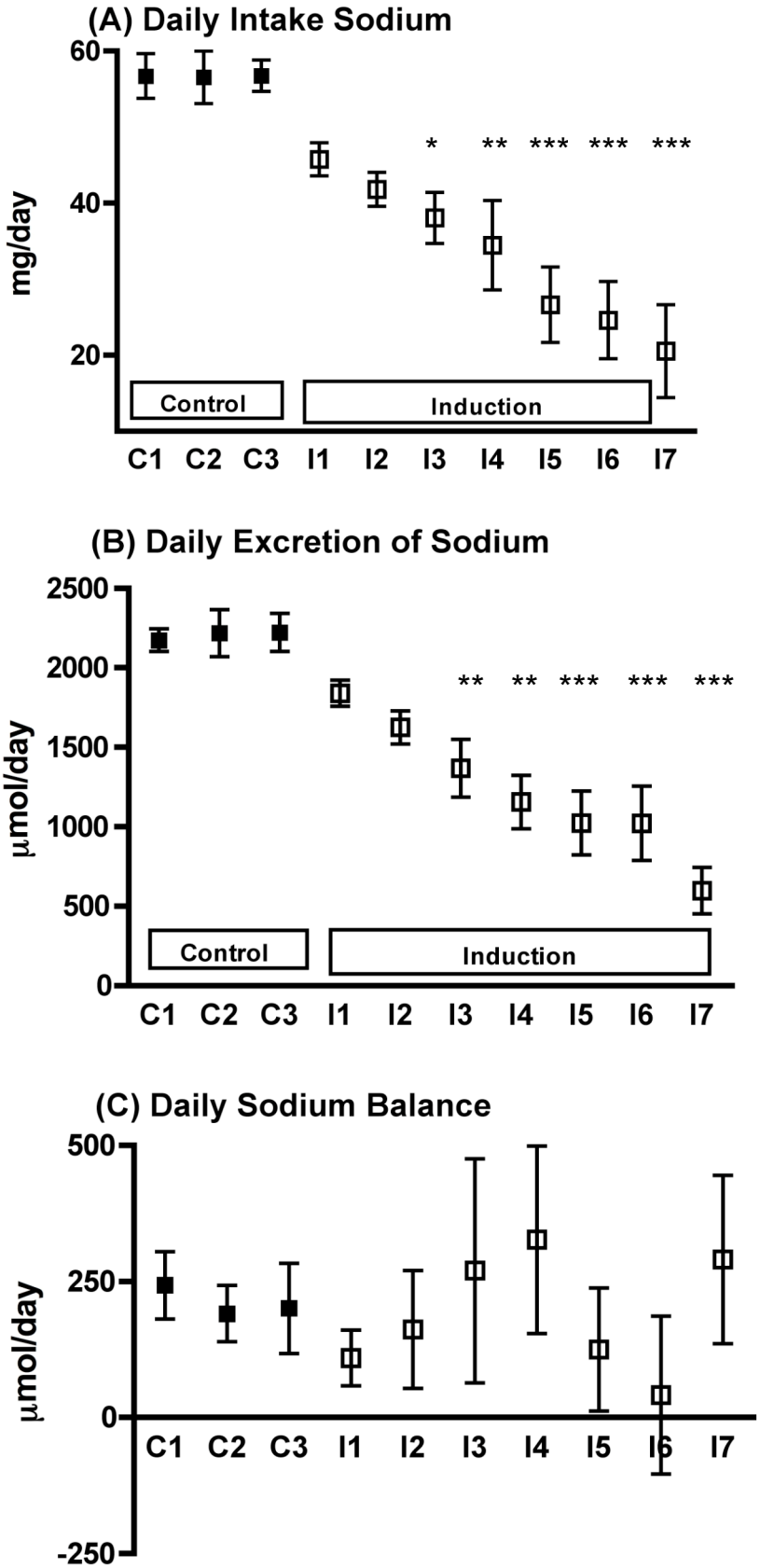


Figure 3-13: Daily measurement of (A) Sodium Intake (B) Sodium Excretion, and (C) Sodium Balance during control period and transgene induction. C1, C2 and C3 represent the control period without any induction. I1 to I7 represent the induction from day 1 to 7. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs mean of control period readings.

3.5 Effect of dietary sodium restriction on Cyp1a1-mRen2 transgene induction.

It has been shown previously that salt-sensitive hypertension develops after transient induction of ANG II-dependent hypertension in this transgenic rat model (Howard *et al.,* 2005). Earlier in the current study, renal haemodynamics were found to be unaltered suggests that enhanced tubular reabsorption might contribute to the maintenance of this form of salt-sensitive hypertension. Moreover an increase in sodium reabsorption was also observed in the transgenic rats following induction. The present study was performed to evaluate the effects of dietary salt restriction on the development of ANG II-dependent hypertension in Cyp1a1-mRen2.F transgenic rat. It has been hypothesized that, under the conditions of an activated RAAS, salt restriction should attenuate the course of hypertension. For the current study a group of 5 transgenic rats were maintained on a low sodium diet before and during a 7 day period of induction.

3.5.1 Blood pressure

Systolic blood pressure was measured daily by tail cuff plethysmography. As shown in figure 3-14 no significant change in blood pressure was observed in non-induced Cyp1a1-mRen2.F transgenic rats placed on a low sodium diet. This indicates that basal blood pressure is not salt-sensitive in this model. However induction of mRen2.F still caused a significant rise in blood pressure despite dietary sodium restriction. Although there was a significant increase in the systolic blood pressure of rats kept on a low sodium diet, this was blunted compared to rats on a normal sodium

diet (figure 3-15A). The partial rescue of the hypertensive phenotype by dietary sodium restriction was also confirmed by direct measurement of arterial blood pressure, made after 7 days of induction (Fig 3-15B).

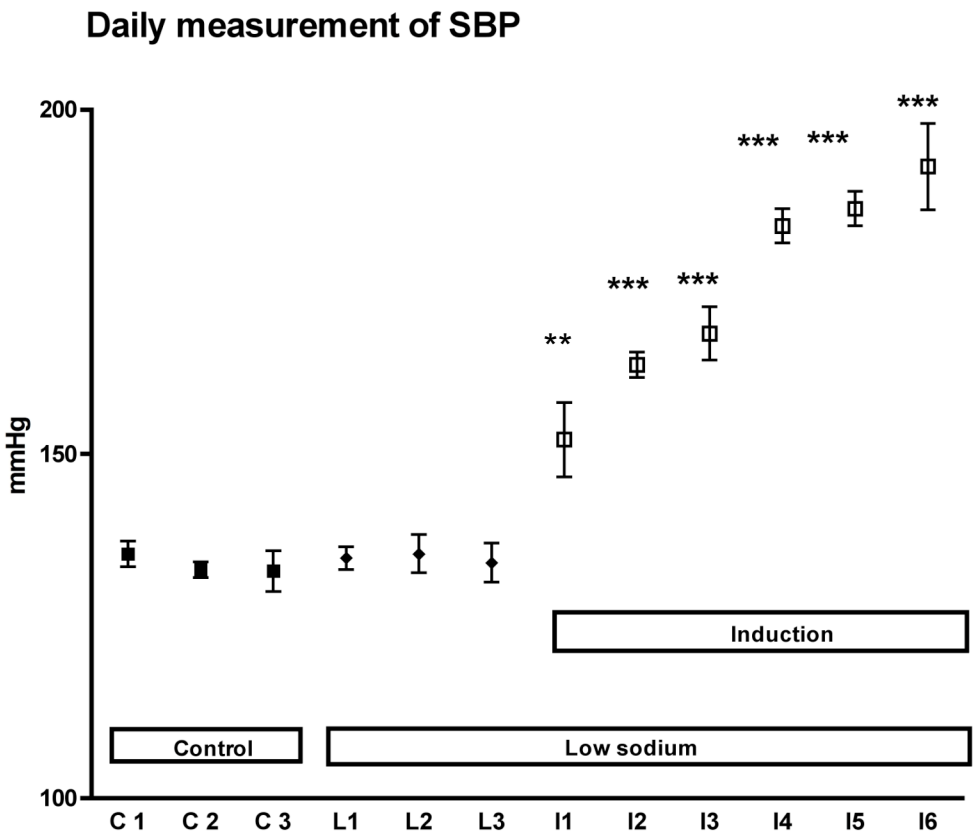


Figure 3-14: Daily measurement of Systolic Blood Pressure by tail cuff plethysmography (n=5). C1 to C3 represent the control period without any induction and rats were kept on normal diet. L1 to L3 represent the time period when rats were kept on low sodium diet without any induction. I1 to I6 represent the induction period from day 1 to 6 when animals were kept on low sodium diet. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. **=$p<0.01$ and ***=$p<0.001$ vs control period reading.

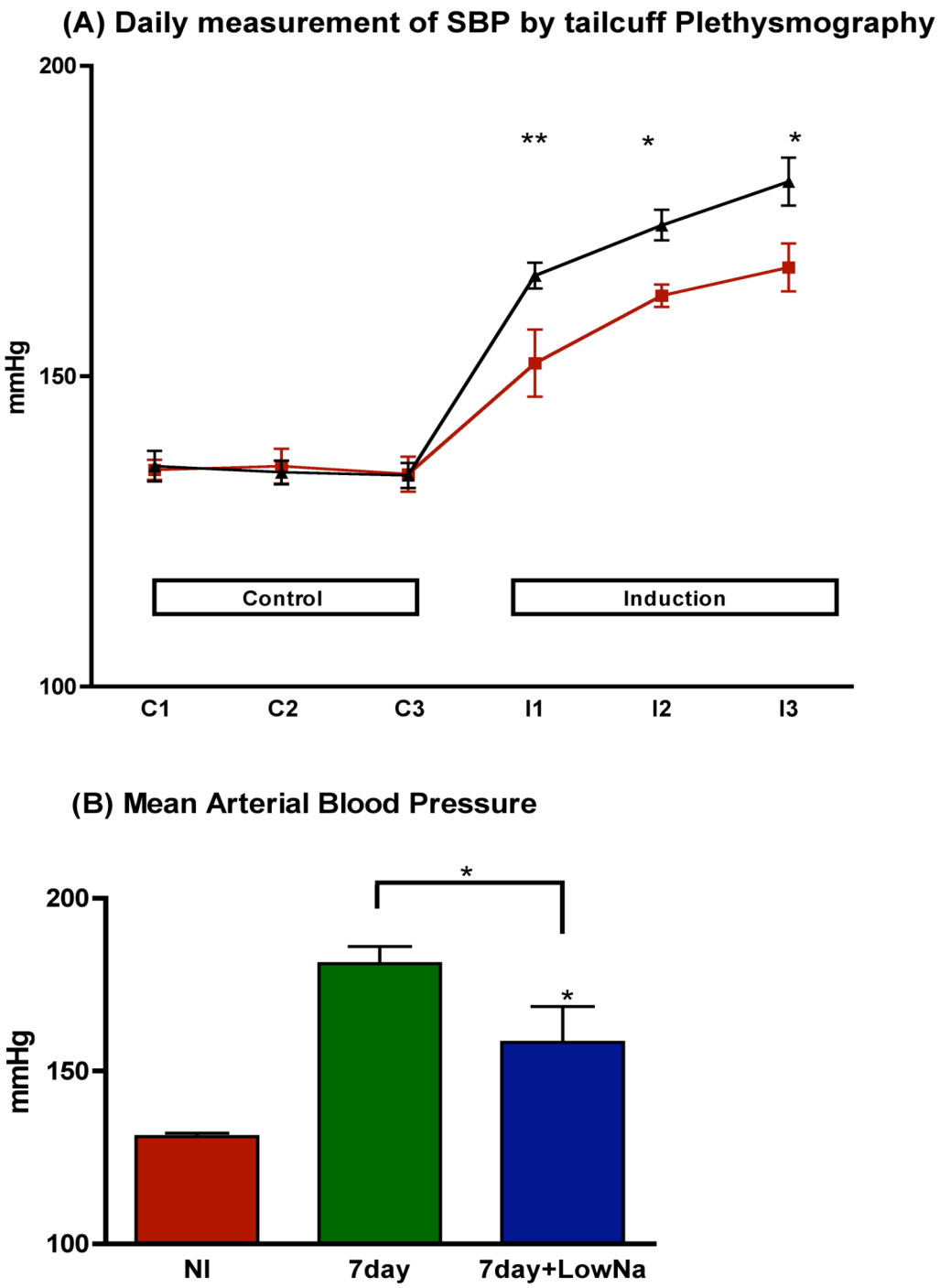


Figure 3-15: (A) Comparison of Systolic Blood Pressure by tail cuff plethysmography between rats kept on normal sodium diet (Black line) and Low sodium diet (Red line). C1 to C3 represent the control period without any induction and I1 to I3 represent the induction period from day 1 to 3. Data are mean±SE and statistical comparisons were made by two way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and **=$p<0.01$.

(B) Mean Arterial Blood Pressure of non-induced control (NI, n=8), 7 day induced normal sodium diet (7day, n=8) and 7 day induced on low sodium diet (7day+LowNa, n=5) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ vs NI group.

3.5.2 Renal haemodynamics

Figure 3-16 shows the GFR, RPF and filtration fraction of non-induced rats, 7-day induced rats on a normal sodium diet and 7-day induced rats kept on a low sodium diet. There was a tendency of elevation of GFR in 7-day induced rats kept on a low sodium diet but this was not statistically significant compare to the non-induced rats. RPF was found to be significantly reduced when induction was carried out together with dietary sodium restriction. In consequence, filtration fraction was elevated in sodium restricted 7-day induced rats.

3.5.3 Urinary sodium excretion

As expected dietary sodium restriction resulted in a further reduction in sodium excretion, but this was not statistically different from 7-day induced rats on a normal diet (Figure 3-17A). The contribution of ENaC and NCC, to the enhanced sodium reabsorption were also measured in this experiment and compared with that of 7 day induced data (ENaC and NCC mediated sodium reabsorption is investigated in chapter 4). The natriuretic effect of amiloride was comparable between all groups (Figure 3-17B). However the natriuretic effect of thiazide decreased significantly from that of 7-day induced rats on normal diet and became comparable with non-induced group (Figure3-17C). This suggests that the up regulation of NCC mediated sodium reabsorption following induction (shown in chapter 4) is normalized by dietary sodium restriction.

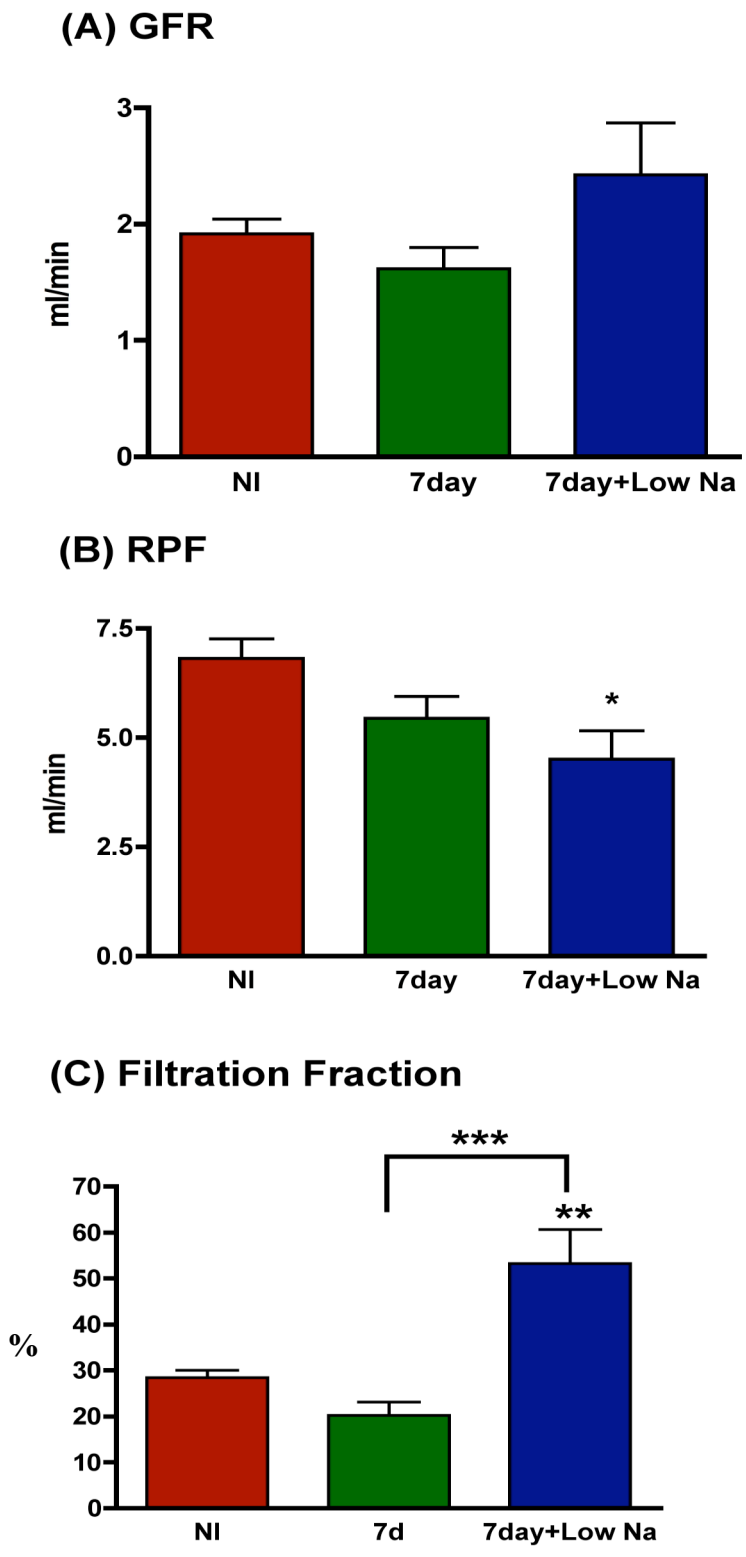


Figure 3-16: (A) GFR, (B) RPF, and (C) Filtration Fraction of non-induced control (NI, n=8), 7-day induced normal sodium diet (7day, n=8) and 7-day induced on low sodium diet (7day+LowNa,, n=5) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs NI group.

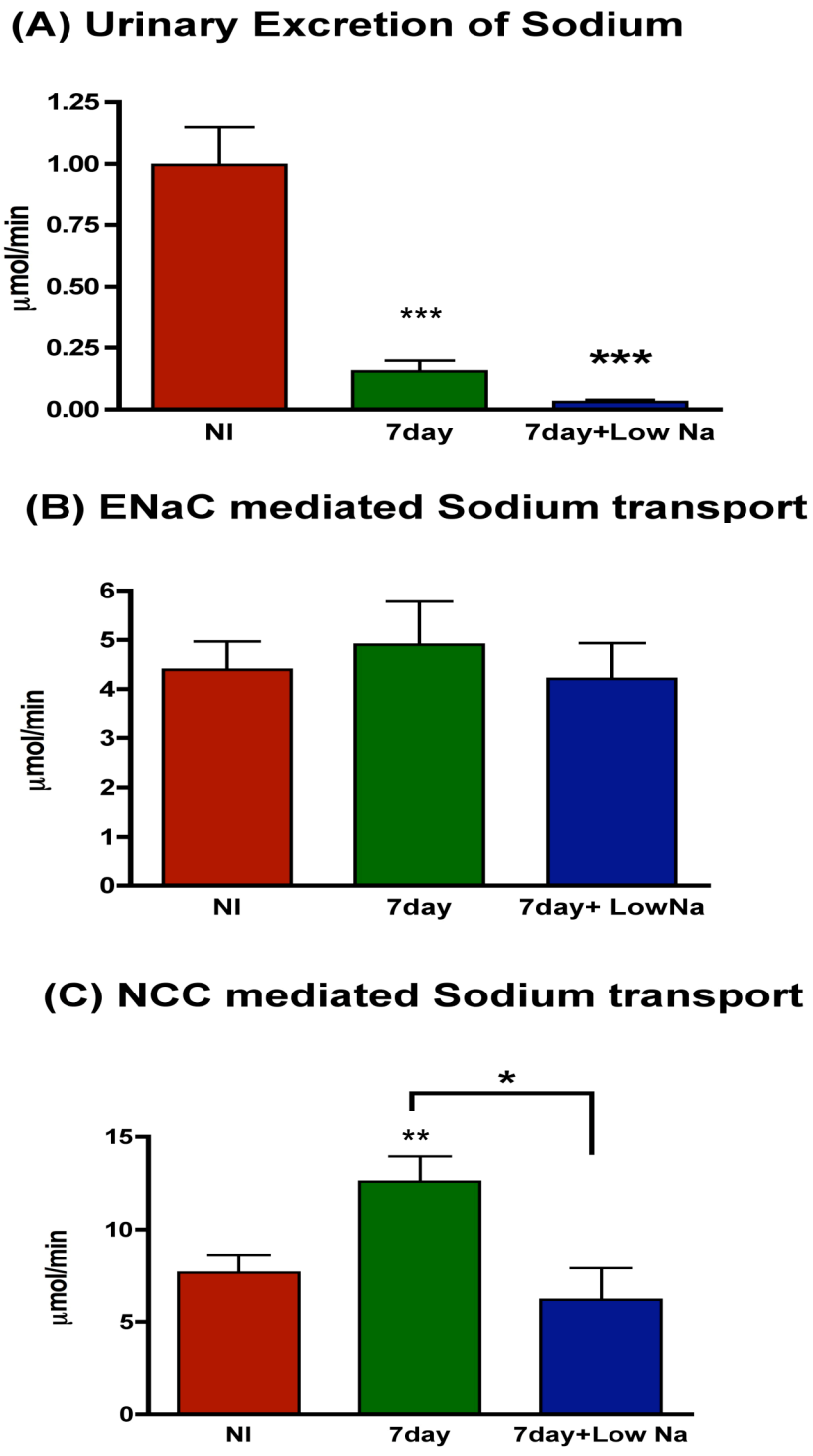


Figure 3-17: (A) Urinary Sodium Excretion (B) ENaC mediated Sodium transport and (C) NCC mediated Sodium transport of non-induced control (NI, n=8), 7-day induced normal sodium diet (7day, n=8) and 7-day induced on low sodium diet (7day+Low Na, n=5) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$, ***=$p<0.01$ and ***=$p<0.001$ vs NI group.

Chapter 4

Mechanisms in the development of

Cyp1a1-mRen2 induced hypertension

4.1 Contribution of the ENaC and NCC to Cyp1a1-mRen2 induced hypertension

A sustained increase in sodium reabsorption was observed following transgene induction. The studies in this chapter addressed the mechanism responsible for the enhanced sodium reabsorption. As renal haemodynamics remained unchanged following induction it was hypothesized that an alteration in tubular sodium transport was responsible for the increased sodium reabsorption. Sodium is freely filtered through the glomerulus, most of the filtered load is reabsorbed along the nephron. The majority of filtered load is reabsorbed in the proximal tubule and loop of Henle. But the sodium transporting proteins in the distal nephron segments are responsible for the fine-tuning of final sodium excretion. Sodium reabsorption in this segment of the nephron is mainly mediated through NCC and ENaC. Several studies have shown that ANG II stimulates the distal sodium transport process by increasing the activity of several sodium transporting proteins such as NHE, NCC, ENaC. In the present study, ENaC and NCC, two sodium transporting proteins in the aldosterone-sensitive distal nephron were targeted to identify their contribution to enhanced sodium reabsorption in Cyp1a1-mRen2.F transgenic rat. ENaC and NCC were blocked acutely by amiloride and thiazide (described in the Methods section) respectively in four different groups of rats. The first group of rats were not induced, the second group of rats were induced for 1 days, the third group of rats were induced for 3 days and the fourth group of rats were induced for 7 days.

4.1.1 Mean arterial blood pressure

Figure 4-1 shows the effect of acute inhibition of ENaC and NCC on MABP in the four different groups. In the non-induced group MABP was not affected by acute administration of either amiloride or amiloride in combination with thiazide. In rats that had been induced for 1 or 7 days, there was a small but significant drop in blood pressure was observed following the combined administration of amiloride and thiazide. Since no difference was observed after amiloride administration, this reduction was attributed to thiazide. Similarly in the 3-day induced group, a reduction in blood pressure was observed following inhibition of ENaC and NCC, but this change did not reach statistical significance.

4.1.2 Renal haemodynamics

Figure 4-2 shows the change in GFR following acute blockade of ENaC and NCC in four different groups of rats. GFR was not affected by acute administration of either amiloride or amiloride in combination with thiazide. However an overall significant difference in GFR was observed in different induction period. The effect of blocking ENaC and NCC on RPF in four different groups are presented in Figure 4-3. Similarly, RPF was not affected by acute administration of either amiloride or amiloride in combination with thiazide in all four groups of rats. Therefore these data suggest that the small drop in blood pressure observed in Figure 4-1 did not influence renal function, since the acute administration of amiloride and thiazide had no effect on either GFR or RPF in any of the groups of rats.

Two-way ANOVA	
Induction period	p<0.0001
Drug effect	p<0.0001
Interaction	ns

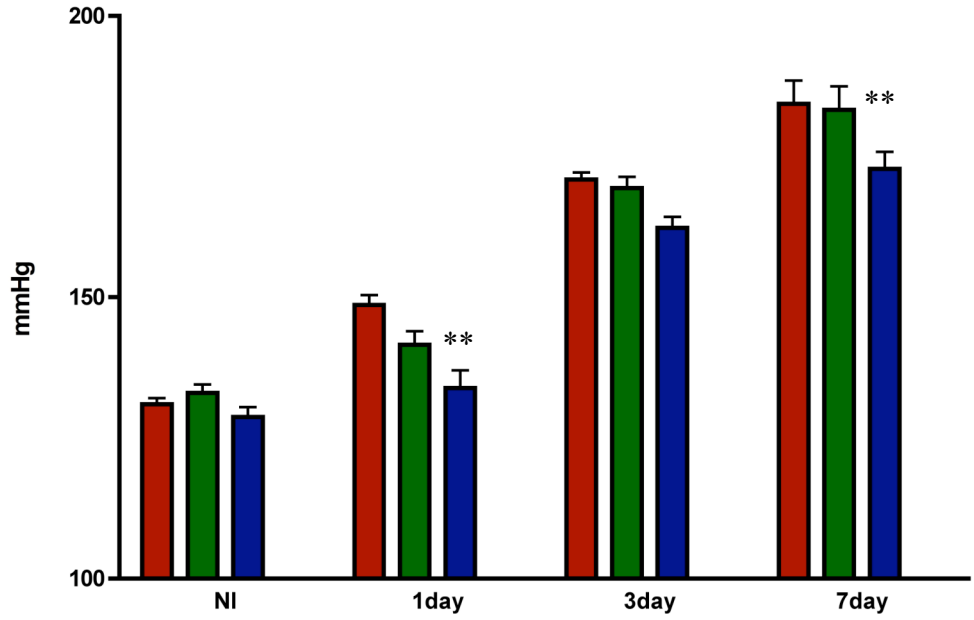


Figure 4-1: Mean Arterial Blood Pressure during 3 different time point of renal clearance study in Non-induced group (NI, n=8), 1-day induced group (1day, n=9), 3-day induced group (3day, n=9) and 7-day induced group (7day, n=8). For each group the red bar represents the measurement without any drug (1st time point), the green bar represents the measurement when ENaC was blocked by amiloride (2nd time point) and the blue bar represents the measurement when both ENaC and NCC were blocked with amiloride and thiazide (3rd time point). Data are mean±SE and statistical comparisons were made by two-way ANOVA with Bonferroni's post-hoc analysis. **=p<0.01 vs no drug period of respective group.

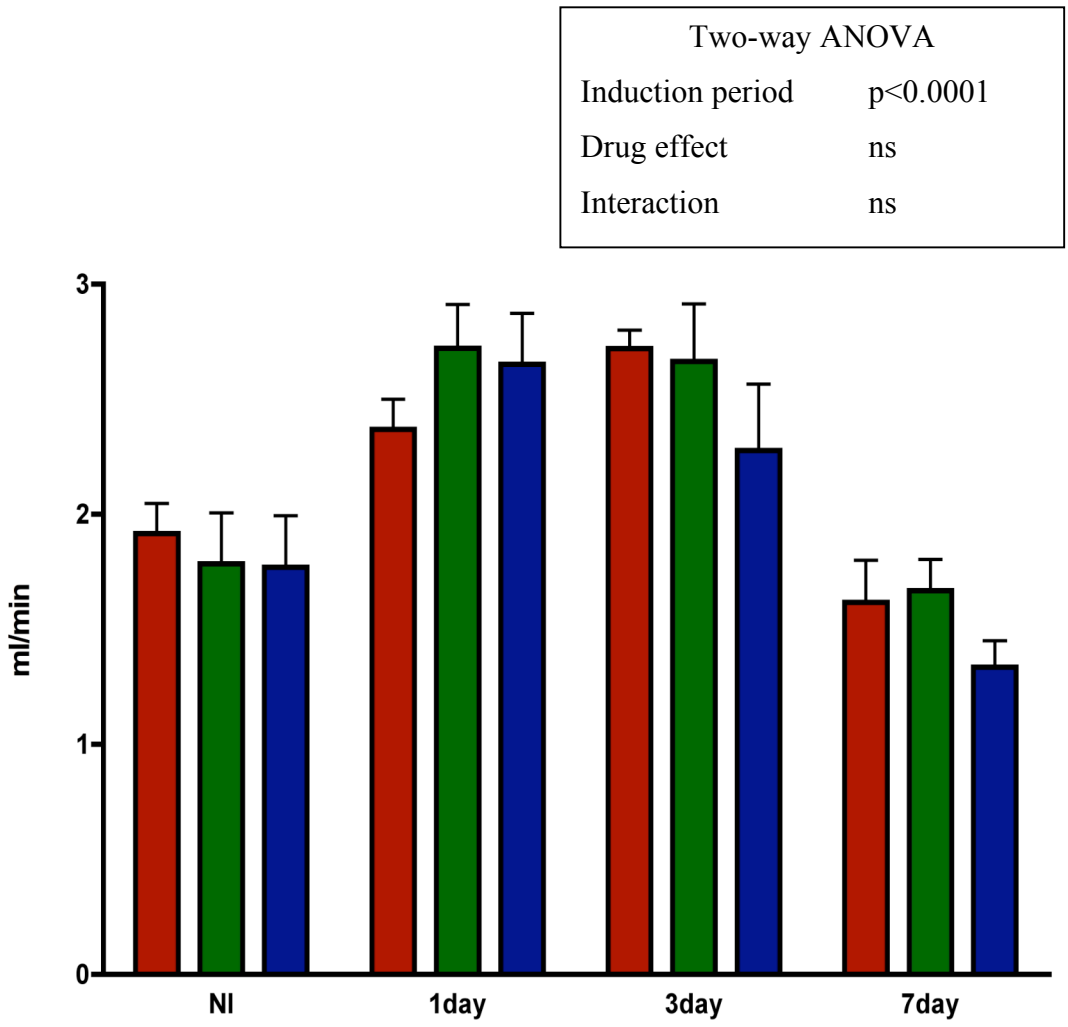


Figure 4-2: Glomerular Filtration Rate during 3 different time point of renal clearance study in Non-induced group (NI, n=8), 1-day induced group (1day, n=9), 3-day induced group (3day, n=9) and 7-day induced group (7day, n=8). For each group the red bar represents the measurement without any drug (1st time point), the green bar represents the measurement when ENaC was blocked by amiloride (2nd time point) and the blue bar represents the measurement when both ENaC and NCC were blocked with amiloride and thiazide (3rd time point). Data are mean±SE and statistical comparisons were made by two-way ANOVA with Bonferroni's post-hoc analysis.

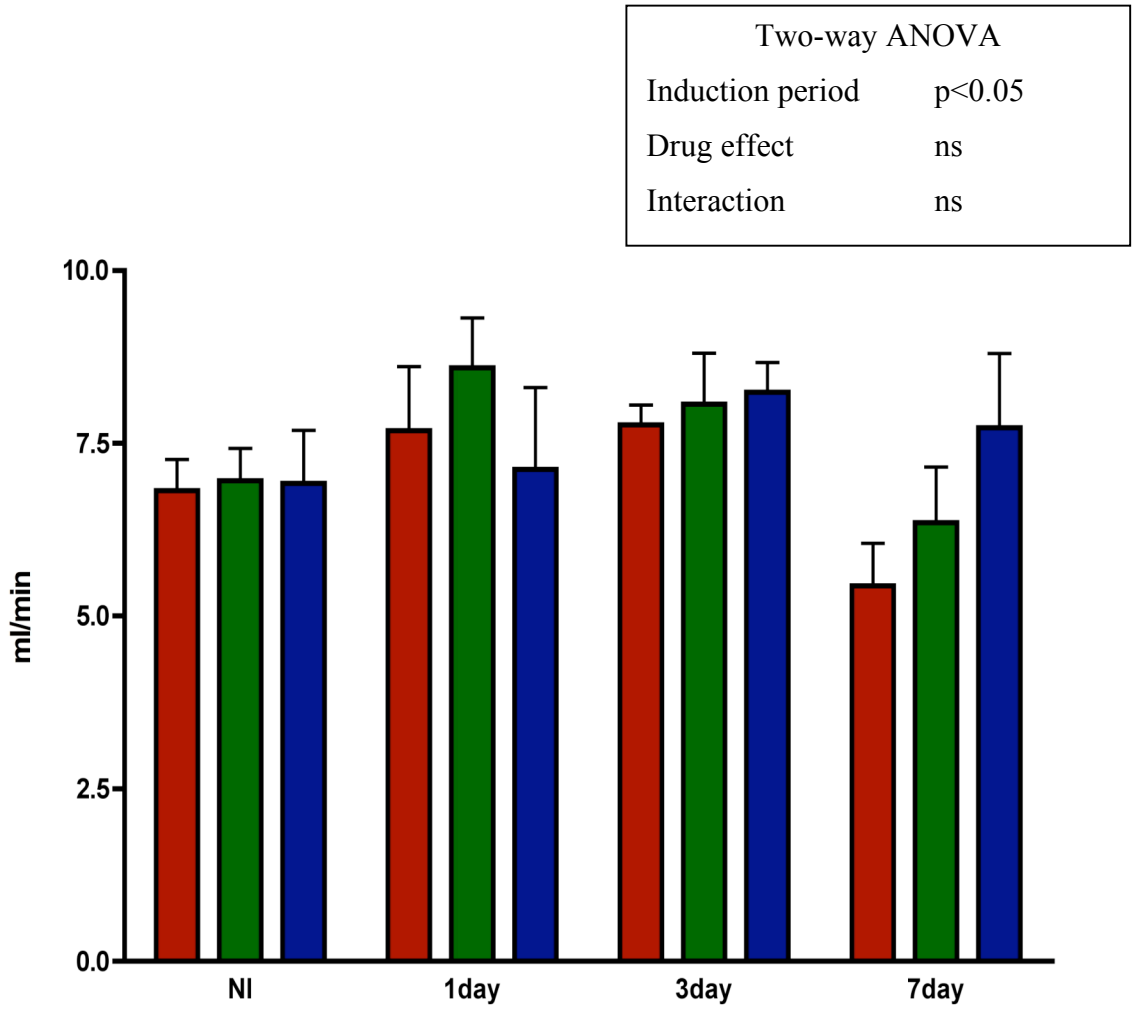


Figure 4-3: Renal Plasma Flow during 3 different time point of renal clearance study in Non-induced group (NI, n=8), 1-day induced group (1day, n=9), 3-day induced group (3day, n=9) and 7-day induced group (7day, n=8). For each group the red bar represents the measurement without any drug (1st time point), the green bar represents the measurement when ENaC was blocked by amiloride (2nd time point) and the blue bar represents the measurement when both ENaC and NCC were blocked with amiloride and thiazide (3rd time point). Data are mean±SE and statistical comparisons were made by two-way ANOVA with Bonferroni's post hoc analysis.

4.1.3 Renal sodium handling

The effect of amiloride and thiazide on renal sodium excretion is summarised in Figure 4-4. Acute blockade of ENaC by amiloride caused a significant increase in sodium excretion in all 4 groups of rats. A further significant increase in sodium excretion was observed when both ENaC and NCC were blocked simultaneously by amiloride and thiazide respectively. Indeed, the effect of thiazide on sodium excretion in all the 4 groups of rats was greater than that of amiloride. This is consistent with the greater role of NCC in renal sodium reabsorption.

To distinguish the individual effects of amiloride and thiazide, the net effect of each drug was calculated by subtraction. The net effect of amiloride on sodium (Δamiloride) represents the amount of sodium reabsorbed via ENaC only. Similarly Δthiazide represents the amount of sodium transported by NCC. In order to prevent downstream compensation by ENaC, sodium reabsorption by NCC was evaluated as the difference between the effect of amiloride and amiloride plus thiazide, rather than using just thiazide alone. Since this may cause an up regulation of ENaC due to increased distal sodium delivery.

Figure 4-5A shows the comparative natriuretic effect of ENaC blockade by amiloride in four different groups. ENaC mediated sodium transport was higher in both 1-day and 3-day induced groups, however these increases were not statistically significant. This indicates that ENaC mediated sodium reabsorption was not responsible for increased sodium retention. NCC mediated sodium transport was also stimulated following the induction of the Cyp1a1-mRen2 transgene (Figure 4-5B). The

enhanced sodium reabsorption via NCC was found statistically significant in 3-day and 7-day induced groups. This implies that an up regulation of NCC occurred following induction and this plays a key role in the increased sodium retention observed following the induction.

Two-way ANOVA	
Induction period	ns
Drug effect	p<0.0001
Interaction	p<0.05

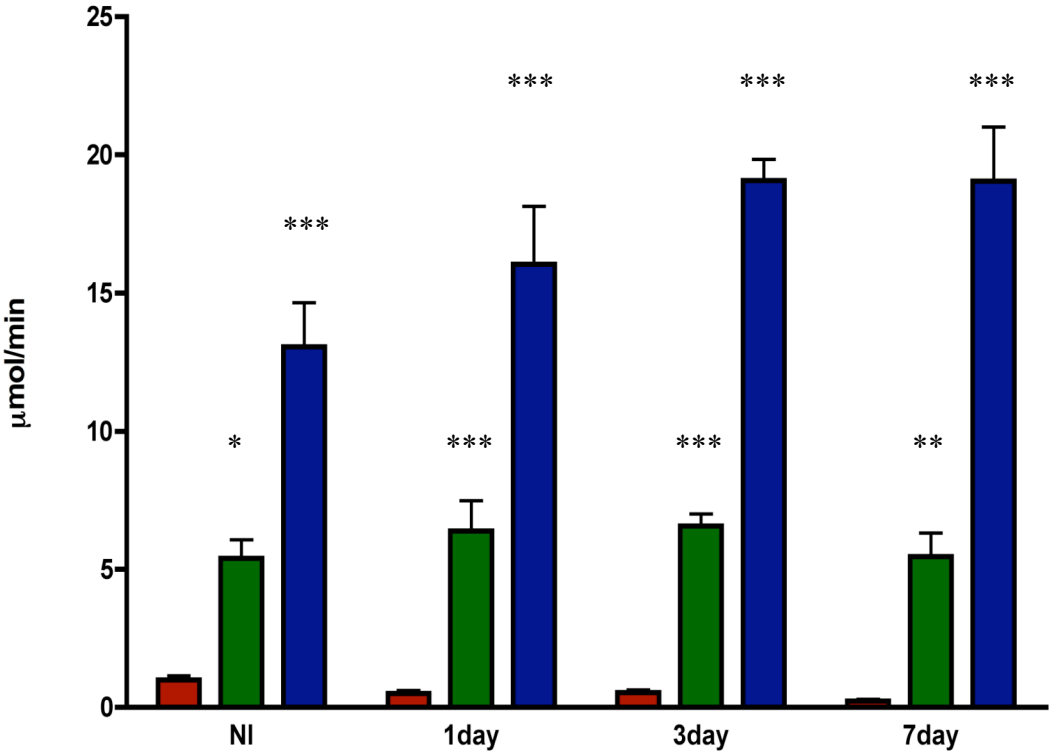


Figure 4-4: Urinary Excretion of Sodium during 3 different time point of renal clearance study in Non-induced group (NI, n=8), 1-day induced group (1day, n=9), 3-day induced group (3day, n=9) and 7-day induced group (7day, n=8). For each group the red bar represents the measurement without any drug(1st time point), the green bar represents the measurement when ENaC was blocked by amiloride (2nd time point) and the blue bar represents the measurement when both ENaC and NCC were blocked with amiloride and thiazide (3rd time point). Data are mean±SE and statistical comparisons were made by two-way ANOVA with Bonferroni's post hoc analysis. *=p<0.05, **=p<0.01 and ***=p<0.001 vs no drug period of respective group.

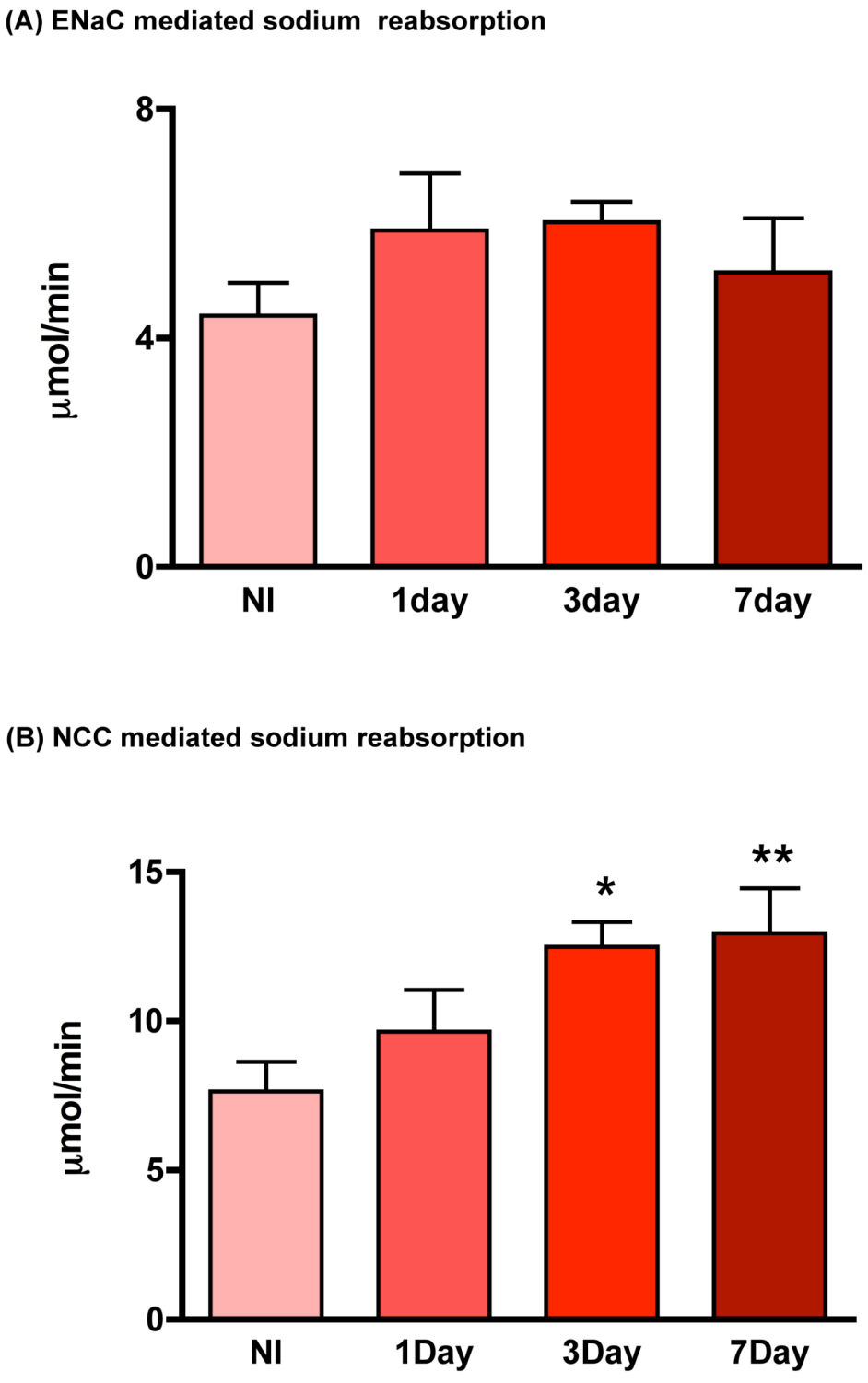


Figure 4-5: (A) ENaC mediated sodium transportation and (B) NCC mediated sodium transportation in non-induced (NI, n=8), 1-day induced (1day, n=9), 3-day induced (3day, n=9) and 7-day induced (7day, n=8) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and **=$p<0.01$ vs NI group.

4.2 Western blot analysis of ENaC and NCC

The main objective of this study was to examine whether the changes observed during acute pharmacological blockade were accompanied by similar changes in protein abundance. The expression of two transporters, ENaC and NCC were determined by semiquantitative assessment of Western blot analysis. In addition, expression of Sgk1 and Nedd4 were also studied for their predominant role in the regulation of ENaC.

4.2.1 Optimisation of extraction of protein from whole kidney

Initially for Western blot analysis study, whole kidney protein was extracted using lysis buffer 1 (see Methods section for detail). A gel was loaded with extracted protein samples and the blot was probed with T4 antibody, which recognized both NKCC1 and NKCC2. NKCC1 and NKCC2 are the two isoforms of the Na-K-Cl cotransporter (NKCC), abundant in the kidney. A single band of approximately 140 kDa was identified (Figure 4-6A). Subsequently the blot was stripped and re-probed with N1 antibody, specific for NKCC1. This antibody identified a protein of similar size (Figure 4-6B).

When the membrane was probed with a NCC antibody (Alpha Diagnostic), the antibody failed to identify any protein (Figure 4-7A). The same blot was stripped and re-probed with N1 antibody. N1 antibody identified NKCC1 at approximately 140 kDa size (Figure 4-7B). At this point, protein from whole kidney was extracted by using a hypotonic lysis buffer without any detergent (buffer 2, see the Methods

section for detail). This time probing with NCC antibody produced a single band at 130 kDa (Figure 4-7C).

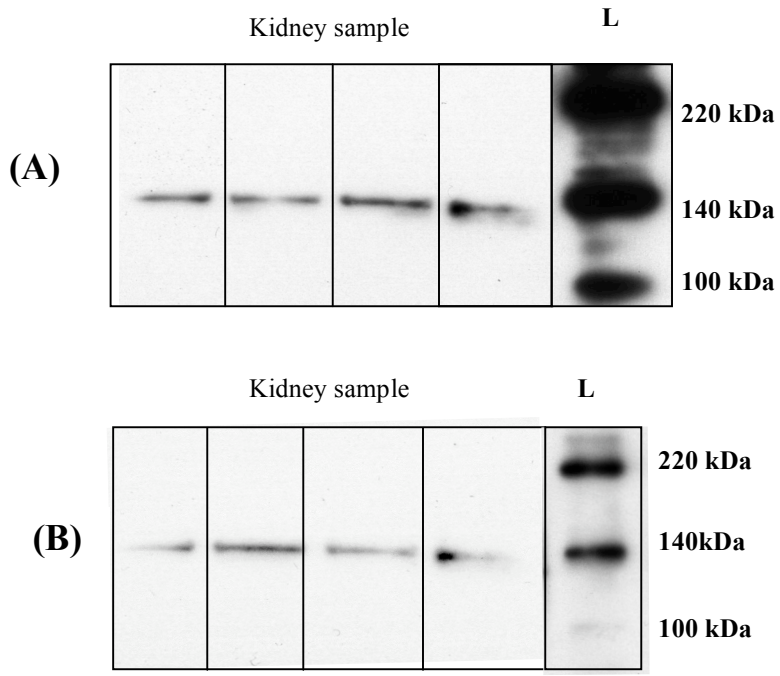


Figure 4-6: (A) Whole kidney protein samples extracted by lysis buffer 1 and probed with T4 antibody. (B) Probed with N1 antibody. L indicates molecular Ladder.

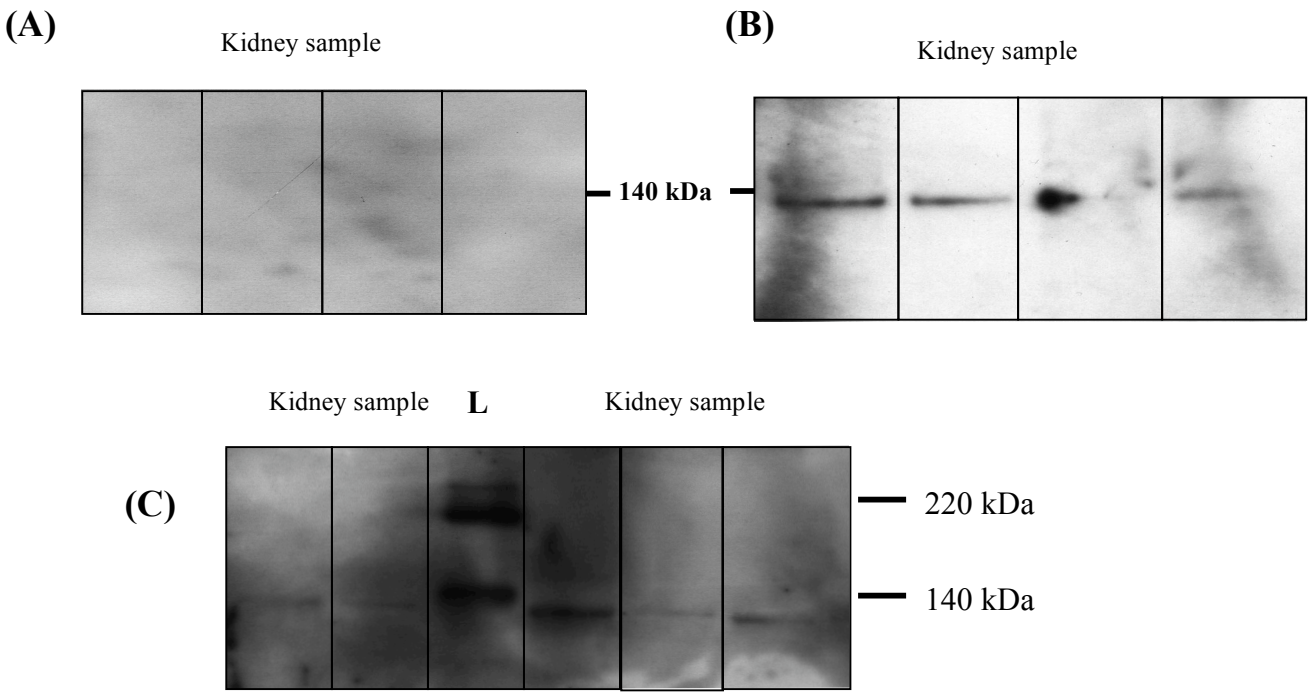


Figure 4-7: (A) Whole kidney protein samples extracted by lysis buffer 1 and probed with NCC (Alpha Diagnostic) antibody. (B) Probed with N1 antibody. (C) Protein samples extracted with lysis buffer 2 and probed with NCC (Alpha Diagnostic) antibody. L indicates molecular Ladder.

4.2.2 Optimization for the NCC antibody

Western blots developed with the NCC antibody from Alpha Diagnostic had very strong background. To reduce this background noise, membranes were blocked with BSA instead of skimmed milk and I also tried to probe with a lower concentration of secondary antibody. However these approaches failed to reduce the background. At this point another NCC antibody (from Chemicon) was used and this antibody identified the same size of protein without background noise. (Figure 4-8A)

To confirm the specificity of the Chemicon antibody, extract from HEK cells was used as a negative control as they do not express NCC. Figure 4-8B shows that no NCC was identified in the HEK cell extract and a single protein was identified in whole kidney extract. Whole kidney extract was also run with a positive control, ferret red blood cell sample, where NCC was identified at 160 kDa, whilst in the whole kidney sample NCC was found to be at 130 kDa (Figure 4-8C).

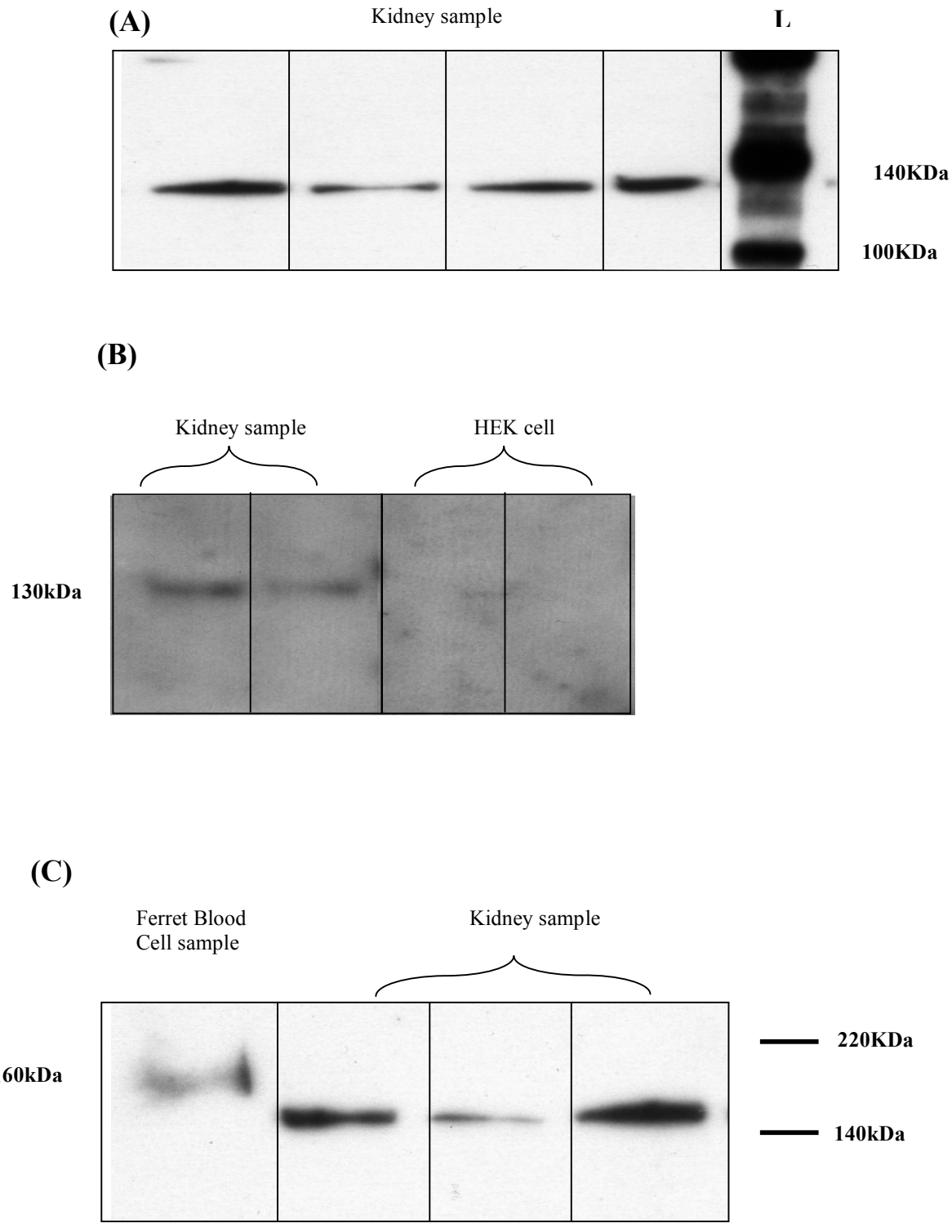


Figure 4-8: (A) Whole kidney protein samples probed with NCC (Chemicon) antibody.(B) HEK cell line extract used as negative control. (C) Ferret red blood cell was used as positive control. L indicates molecular Ladder.

4.2.3 Expression of NCC

The up-regulation of NCC due to transgene induction identified from the pharmacological intervention study was supported by Western blotting findings. Figure 4-9 shows a representative immunoblot and summary of densitometry quantification of the whole kidney NCC of non-induced, 1-day induced, 3-day induced and 7-day induced group. The anti-NCC antibody identified a single protein at approximately 130 kDa. There was a notable difference in NCC level of induced rats compare to non-induced control rats. A significant increase in NCC expression in 3-day and 7-day induced rats was found by densitometry.

As activity of NCC largely depends on phosphorylation (San-Cristobal *et al.*, 2009), whole kidney extract from non-induced, 1-day induced, 3-day induced and 7-day induced groups were probed with an antibody specific for phosphorylated NCC (gift from Professor Dario Alessi, University of Dundee). This identified a band of 130 kDa but no difference in phosphorylated NCC was observed following transgene induction (Figure 4-9C).

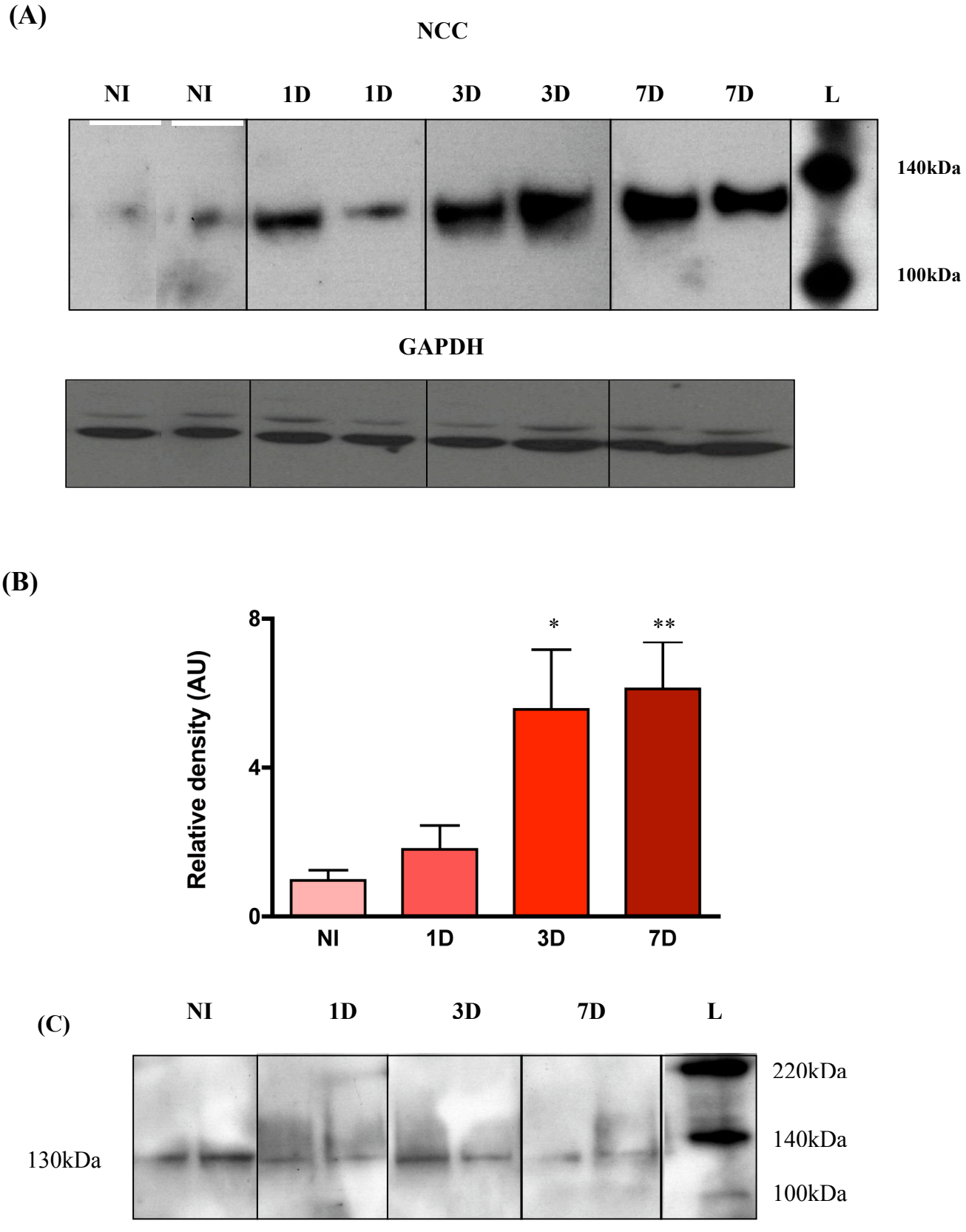


Figure 4-9: Whole kidney NCC protein abundance in Non Induced (NI, n=6), 1day Induced (1D, n=6), 3day Induced (3D, n=6) and 7day Induced (7D, n=6). Western blot analysis identified a single protein at approximately 130 kDa (A). Summary of densitometry quantification of 4 groups of rat is shown in B. Results were adjusted to GAPDH and expressed as relative density (AU= arbitrary units). Abundance of phosphorylated NCC in the whole kidney extract from 4 different groups of rat (C). L indicates molecular Ladder.

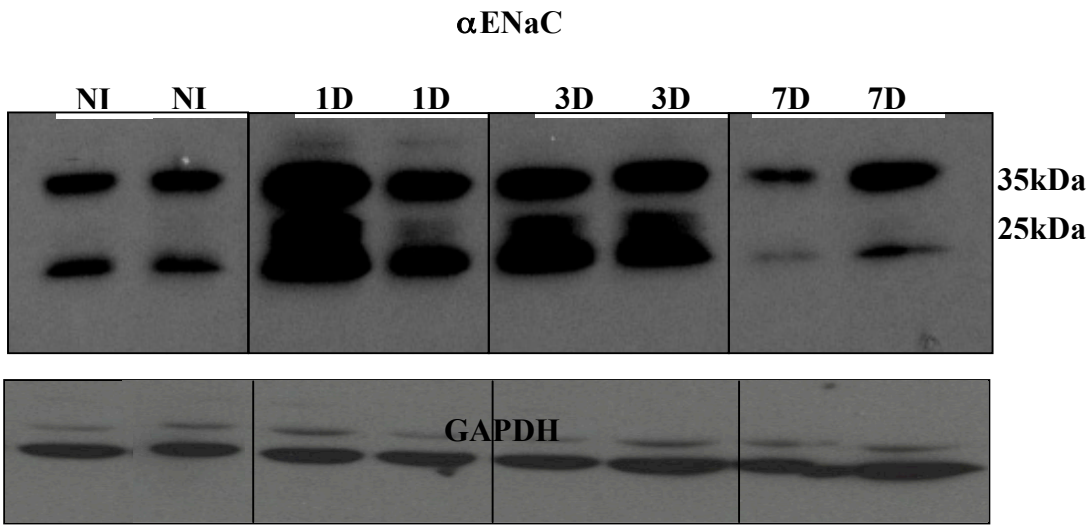
4.2.4 Expression of αENaC

Figure 4-10 shows a representative immunoblot and densitometry quantitation for αENaC in whole kidney of non-induced, 1-day induced, 3-day induced and 7-day induced mRen2.F rats. Two distinct bands at the size of 35 kDa and 25 kDa were identified by the anti-αENaC antibody. There was a tendency for increased expression of ENaC after transgene induction. However this trend was statistically significant only for 1-day induced group. This indicates that there was a transient increase in αENaC expression at the early period of induction. This trend was not observed after either 3 or 7 days of induction.

4.2.5 Expression of Sgk1

Along with ENaC and NCC, the expression of Serum glucocorticoid-inducible kinase (Sgk1) was also measured for its important role in the regulation of epithelial sodium ion transport. A representative immunoblot and summary of densitometry quantitation of the whole kidney Sgk1 of non-induced, 1-day induced, 3-day induced and 7-day induced group is shown in Figure 4-11. The anti Sgk1 antibody identified two distinct proteins at approximately 50 kDa and 30 kDa respectively. Western blot analysis could not find any effect of transgene induction on the abundance of Sgk1 protein.

(A)



(B)

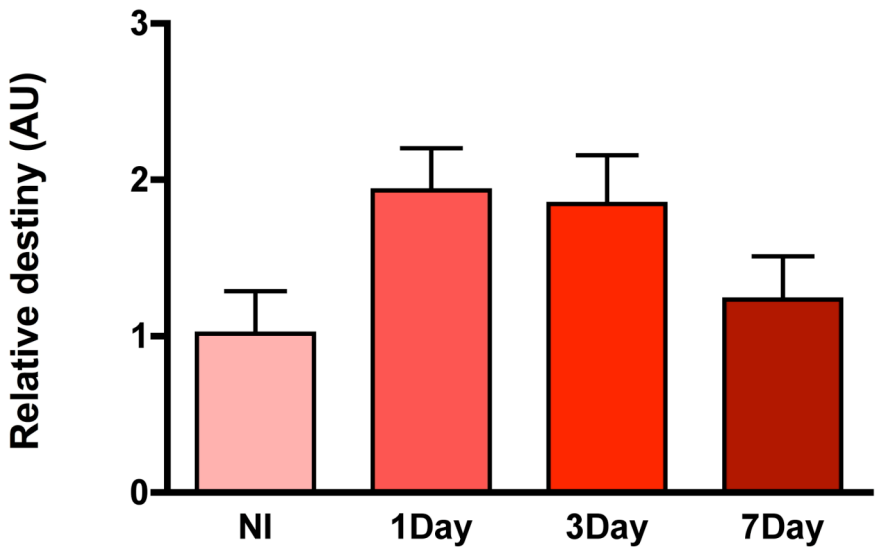
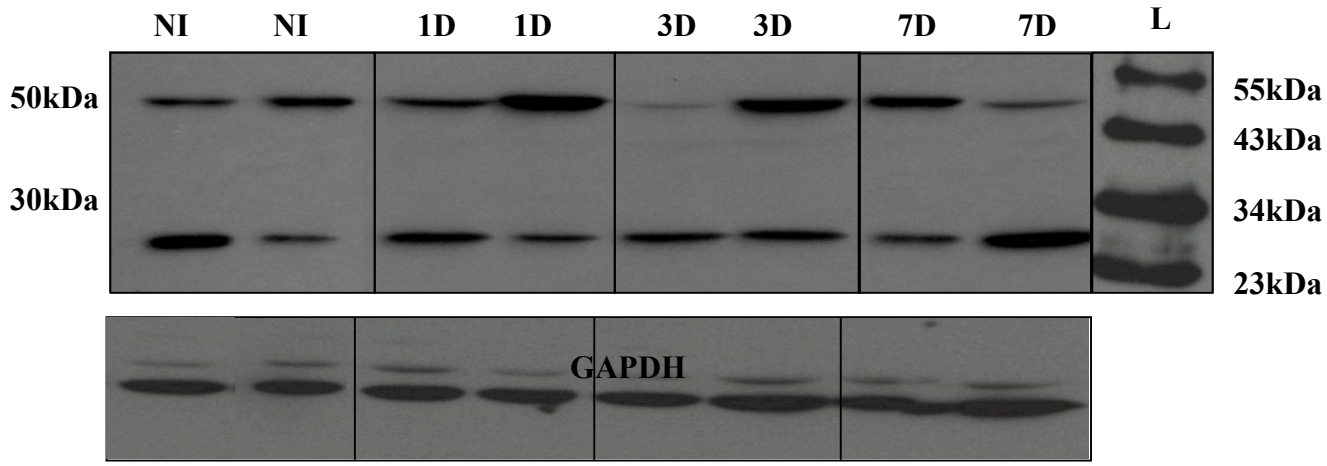


Figure 4-10: Whole kidney αENaC protein abundance in Non Induced (NI, n=6), 1day Induced (1D, n=6), 3day Induced (3D, n=6) and 7day Induced (7D, n=6). Western blot analysis identified two distinct proteins at approximately 35 and 25 kDa (A). Summary of densitometry quantification of αENaC (35 kDa) from 4 groups of rat is shown in B. Results were adjusted to GAPDH and expressed as relative density (AU= arbitrary units).

(A)

Sgk1



(B)

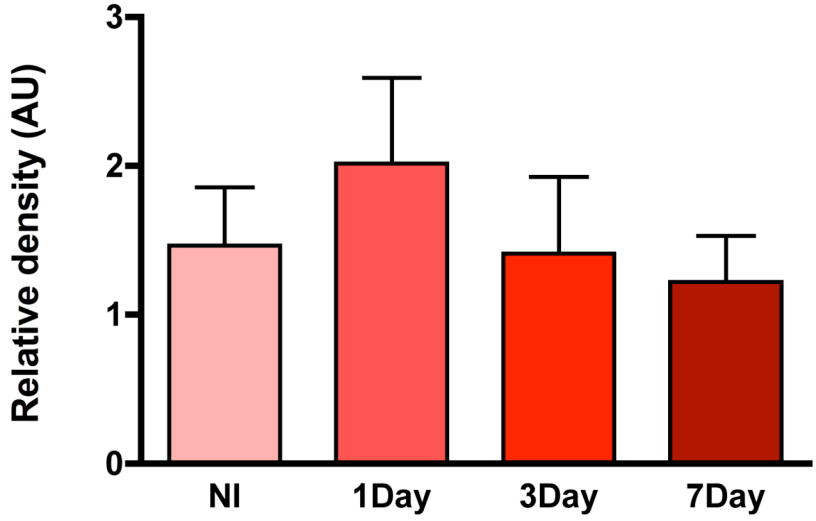
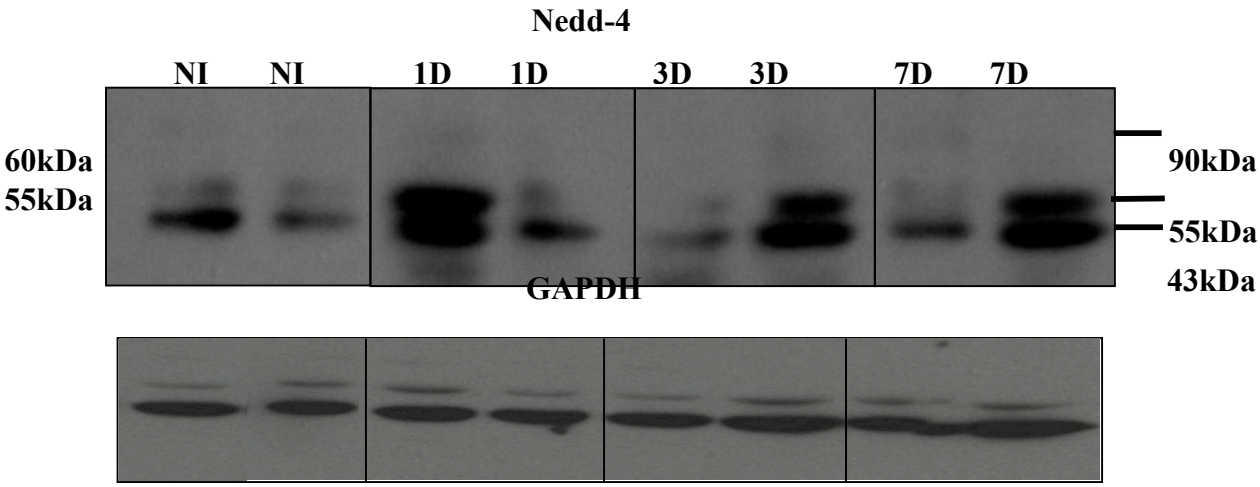


Figure 4-11: Whole kidney Sgk1 protein abundance in Non Induced (NI, n=6), 1day Induced (1D, n=6), 3day Induced (3D, n=6) and 7day Induced (7D, n=6). Western blot analysis identified two distinct proteins at approximately 50 and 30 kDa (A). Summary of densitometry quantification of Sgk1 (50 kDa) from 4 groups of rat is shown in B. Results were adjusted to GAPDH and expressed as relative density (AU= arbitrary units). L indicates molecular Ladder.

The abundance of Nedd-4 was also measured by Western blot analysis for its major role in ENaC activity. The apical targeting of ENaC is controlled by the ubiquitin

ligase, Nedd-4. Figure 4-12 shows the representative blot and a summary of density quantification of whole kidney Nedd-4 from non-induced, 1-day induced, 3-day induced and 7-day induced groups. Two distinct bands at approximately 60 kDa and 55 kDa were identified by anti Nedd-4 antibody. A tendency of higher expression of Nedd-4 was observed in induced animals but this increase was not statistically significant.

(A)



(B)

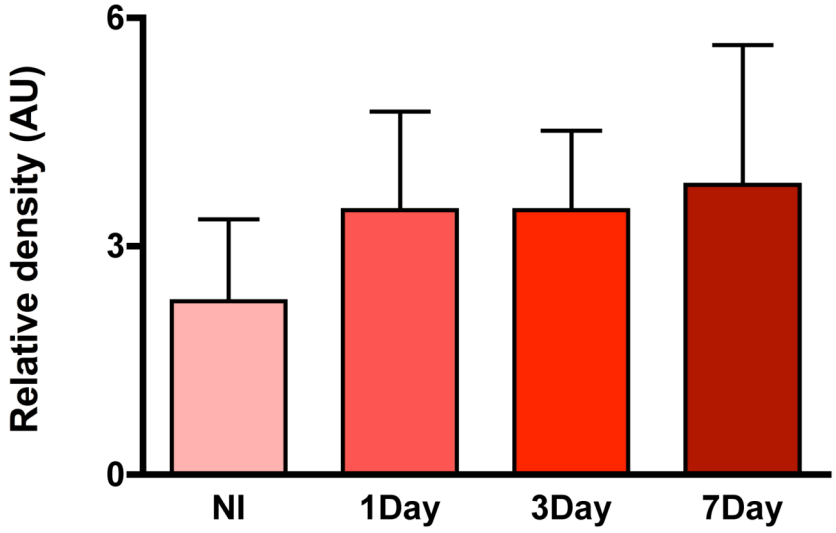


Figure 4-12: Whole kidney Nedd-4 protein abundance in Non Induced (NI, n=6), 1day Induced (1D, n=6), 3day Induced (3D, n=6) and 7day Induced (7D, n=6). Western blot analysis identified two distinct proteins at approximately 60 and 55 kDa (A). Summary of densitometry quantification of Nedd-4 (55 kDa) from 4 groups of rat is shown in B. Results were adjusted to GAPDH and expressed as relative density (AU= arbitrary units).

4.3 Effect of chronic inhibition of NCC

It has been shown previously that NCC-mediated sodium transport was significantly enhanced following the transgene induction for 3 days and 7 days. To assess the role of NCC in the development in hypertension in this ANG II-dependent model, NCC was chronically blocked with thiazide administration by minipump; subsequently induction was carried out for three consecutive days. For control purposes, a group of transgenic rats were used in which vehicle-carrying minipumps were implanted prior to the 3 days of induction.

4.3.1 Effect on blood pressure

Daily SBP was measured by tail cuff plethysmography before minipump implantation, after minipump implantation and during the period of induction. Figure 4-13A shows that thiazide caused no significant change in blood pressure in the transgenic rats before the initiation of induction. Induction resulted in an increase in blood pressure however this increase was blunted in the thiazide-treated group relative to the 3-day induce group (4-13B). After 3 days of induction rats were anaesthetized for MABP measurements and analysis of renal function. MABP was found to be significantly reduced in the thiazide-treated 3-day induced group compare to both the vehicle only group and the 3-day induced group (Figure 4-13C). However the MABP of thiazide-treated 3-day induced group remained higher than that of the non-induced group. Thus chronic inhibition of NCC partially rescues the hypertensive phenotype.

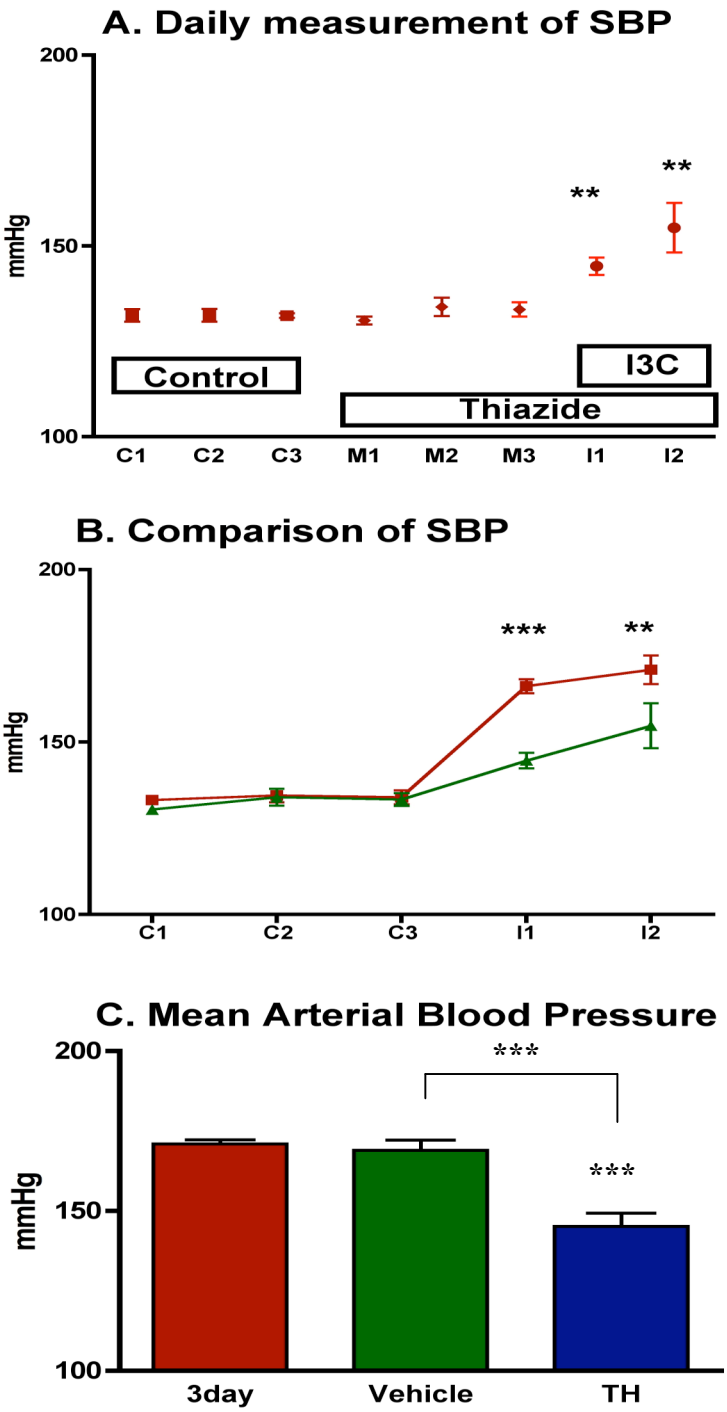


Figure 4-13:(A) Daily measurement of Systolic Blood Pressure by tail cuff (n=6). C1 to C3 represent the control period without any induction, M1 to M3 represent the time period after implantation of minipump carrying thiazide before induction starts. I1 to I2 represent the induction with I3C from day 1 to 2. (B) Comparison of Systolic Blood Pressure by tail cuff between 3-day induced group (Red line) and thiazide treated 3-day induced group (Green line). C1 to C3 represent the control period without any induction and I1 to I2 represent the induction period from day 1 to 2. (C) Mean Arterial Blood Pressure of 3-day induced (3day, n=9), 3-day induced vehicle group (Vehicle, n=6) and thiazide treated 3-day induced group (TH, n=6). Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. **=$p<0.01$ and ***=$p<0.001$ vs 3-day induced group.

4.3.2 Effect on renal haemodynamics

Figure 4-14 shows the GFR, RPF and filtration fraction of the 3-day induced, vehicle only and thiazide treated 3-day induced groups. Thiazide treatment caused a significant reduction in GFR. Similarly a reduction in RPF was observed following thiazide administration, however this did not reach statistical significance. Filtration fraction also decreased following NCC blockade with thiazide but this reduction was not statistically significant.

4.3.3 Effect on renal sodium handling

Urinary sodium excretion was significantly enhanced following chronic inhibition of NCC (Figure 4-15). Notably, the sodium excretion in vehicle only group was comparable with 3-day induced group. Moreover, both ENaC and NCC mediated sodium reabsorption in vehicle only group were comparable with 3-day induced group. This confirmed that the DMSO vehicle did not have any additional effect on the sodium transport in this study. The natriuretic effect of amiloride was found to be significantly higher in thiazide treated 3-day induced group compared to vehicle only group and 3-day induced group. The increased sodium excretion in response to amiloride following chronic thiazide administration suggests a compensatory enhancement of ENaC-mediated sodium reabsorption. A characteristic natriuretic effect of acute NCC blockade was observed in the vehicle-carrying group but no change was observed in the thiazide treated 3-day induced group. This finding confirms that the dose used for chronic inhibition of NCC was sufficient to block its activity.

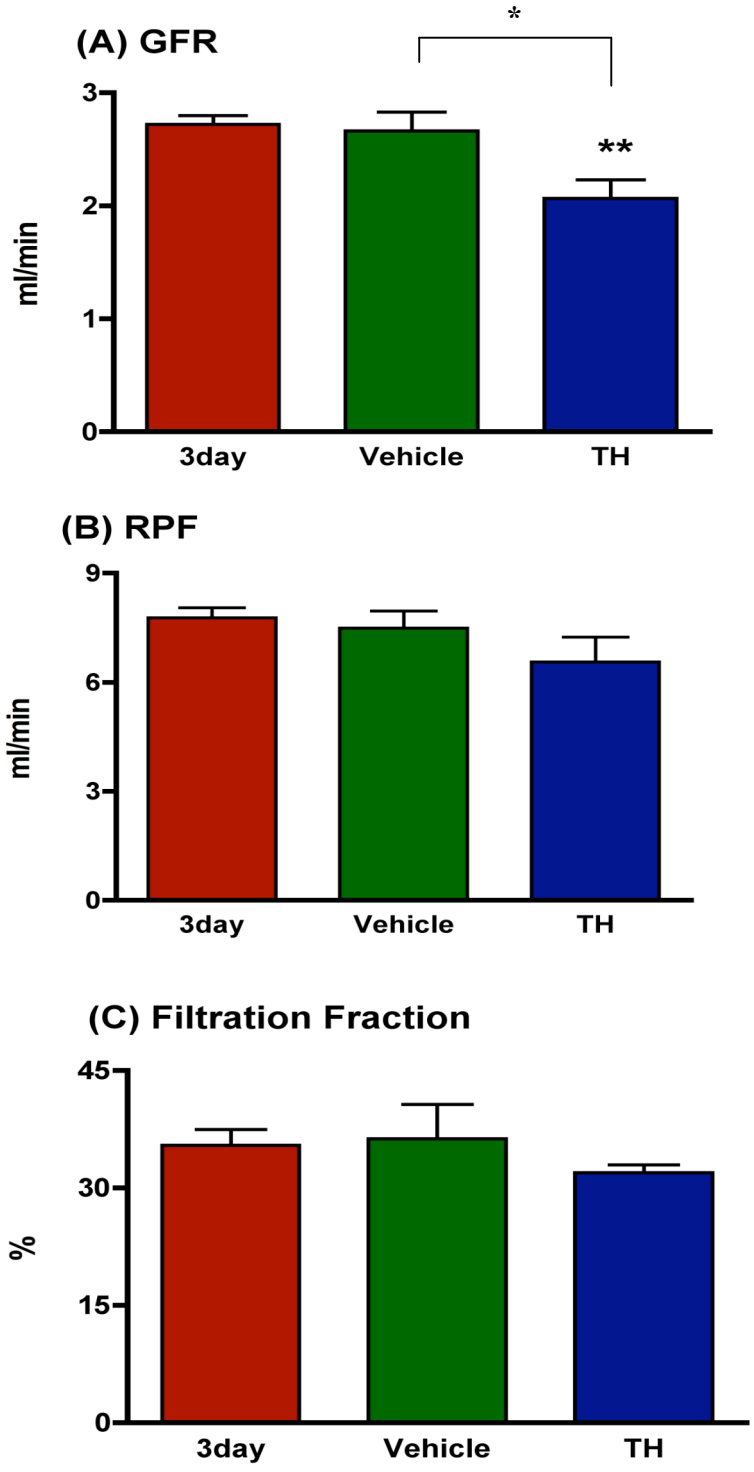


Figure 4-14: (A) GFR, (B) RPF, and (C) Filtration Fraction of 3-day induced (3day, n=9), 3-day induced vehicle group (Vehicle, n=6) and thiazide treated 3-day induced group (TH, n=6). Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and **=$p<0.01$ vs 3-day induced group.

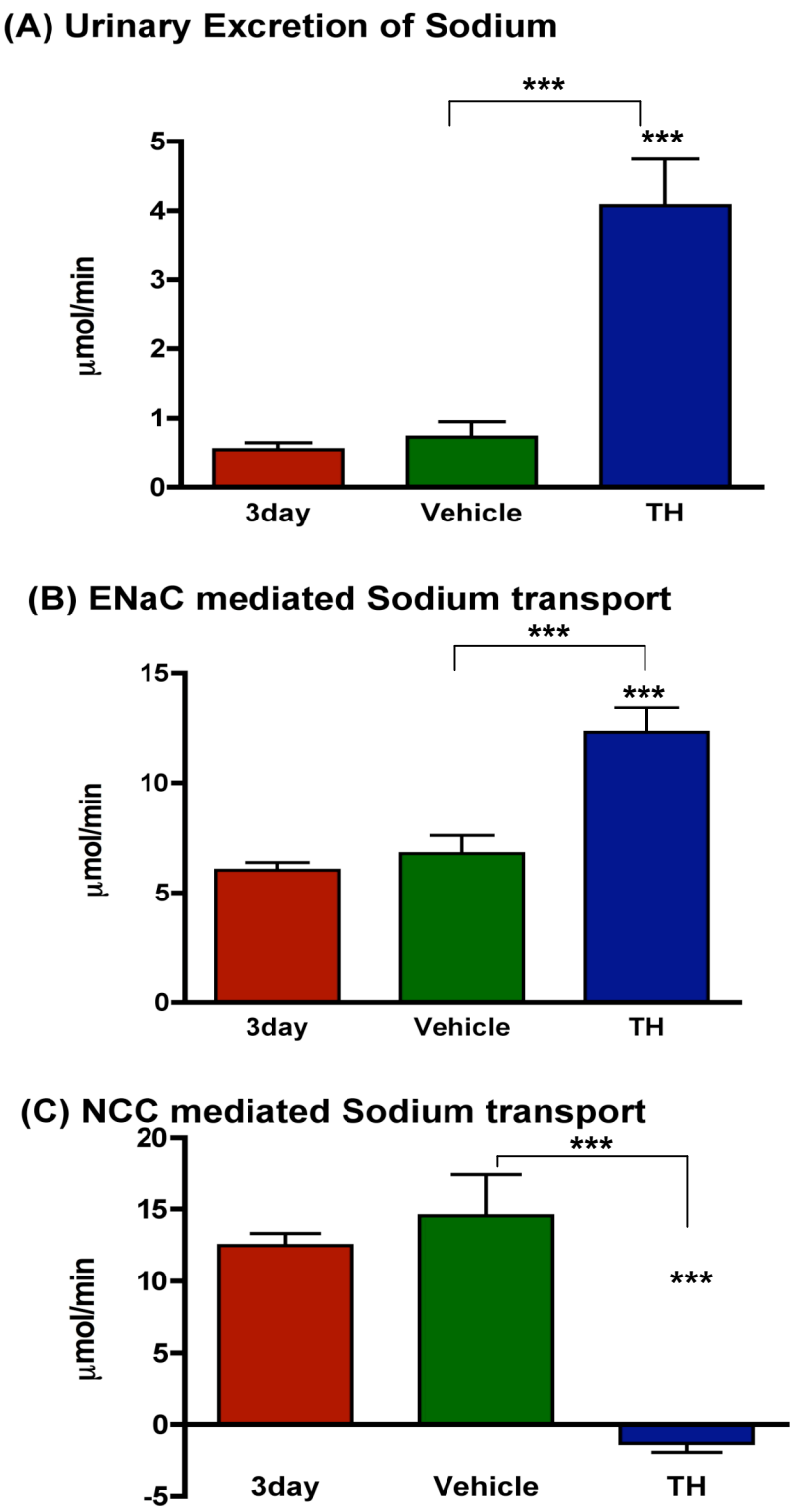


Figure 4-15: (A) Urinary Sodium Excretion (B) ENaC mediated Sodium transport, and (C) NCC mediated Sodium transport of 3-day induced (3day, n=9), 3-day induced vehicle group (Vehicle, n=6) and thiazide treated 3-day induced group (TH, n=6). Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. ***=$p<0.001$ vs 3-day induced group.

4.4 Effect of MR blockade on the development of ANG II-dependent hypertension

4.4.1 Urinary excretion of aldosterone in transgene induction

To investigate the role of aldosterone in ANGII-dependent hypertension, daily urinary aldosterone levels were measured from a group of Cyp1a1-mRen2.F transgenic rats maintained in metabolic cages. In this study, following three days of acclimatization period, control measurements were made over a further three days. Subsequently rats were induced by daily gastric gavage of I3C for seven consecutive days. Figure 4-16 shows the daily urinary aldosterone excretion for the control period and during the seven days of induction. The urinary aldosterone level remained comparable with control period during the first two days of induction. A significant increase was observed from the third day of induction. Moreover it had increased up to 15-fold by day five and remained elevated for the rest of the induction period.

Aldosterone plays a key role in the regulation of distal nephron sodium reabsorption. An elevated plasma level of aldosterone was reported earlier in this transgenic rat model following induction. The current study has shown that urinary aldosterone levels increased up to 15-fold following 5 days of induction. This excessive formation of aldosterone may stimulate distal nephron sodium reabsorption and as a result reduce the excretion of sodium. Aldosterone stimulates the reabsorption of sodium via activation of its receptor. Thus it was hypothesized that chronic blockade

of MR would increase the urinary excretion of sodium and as a consequence abrogate the hypertensive phenotype. To investigate whether the observed alteration in renal sodium retention was mediated by aldosterone, MR was chronically blocked with spironolactone, administrated by minipump, subsequently induction was carried out for three consecutive days.

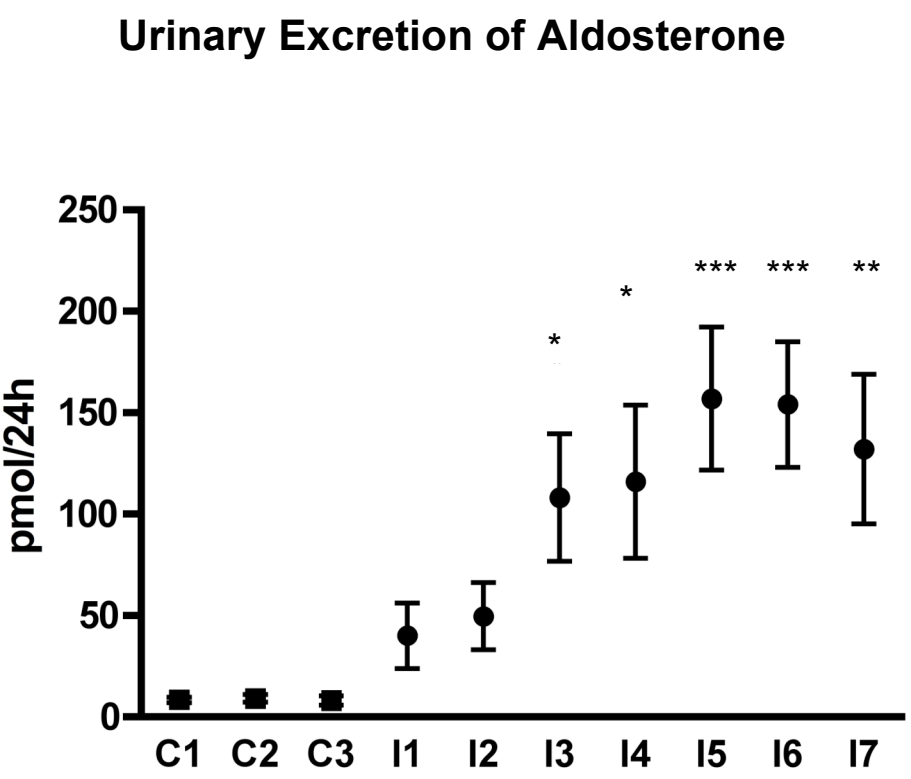


Figure 4-16: Daily measurement of urinary aldosterone excretion during control period and transgene induction. C1, C2 and C3 represent the control period without any induction. I1 to I7 represent the induction from day 1 to 7. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs mean of control period readings.

4.4.2 Effect on blood pressure

To assess the effect of MR blockade on the development of hypertension daily SBP was measured before minipump implantation, after minipump implantation and also during the induction. Figure 4-17 shows that MR blockade had no significant effect on blood pressure in non-induced transgenic rats. Induction resulted in an increase in blood pressure. At the end of the 3 days of induction regime, rats were anaesthetized for MABP measurement and analysis of renal function. MABP of the 3-day induced MR antagonist treated group was significantly elevated compare to non-induced control group and this was comparable with MABP of 3-day induced group (Figure 4-18A). These data indicate that MR blockade failed to rescue the hypertensive phenotype.

4.4.3 Effect on renal haemodynamics

Figure 4-18 shows the GFR and RPF of non-induced, 3-day induced and MR blockade 3-day induced groups. Both GFR and RPF of MR blockade 3-day induced group were comparable with non-induced control however they were significant reduced relative to 3-day induced group. Therefore MR blockade could not rescue the hypertensive phenotype but it normalized the GFR and RPF.

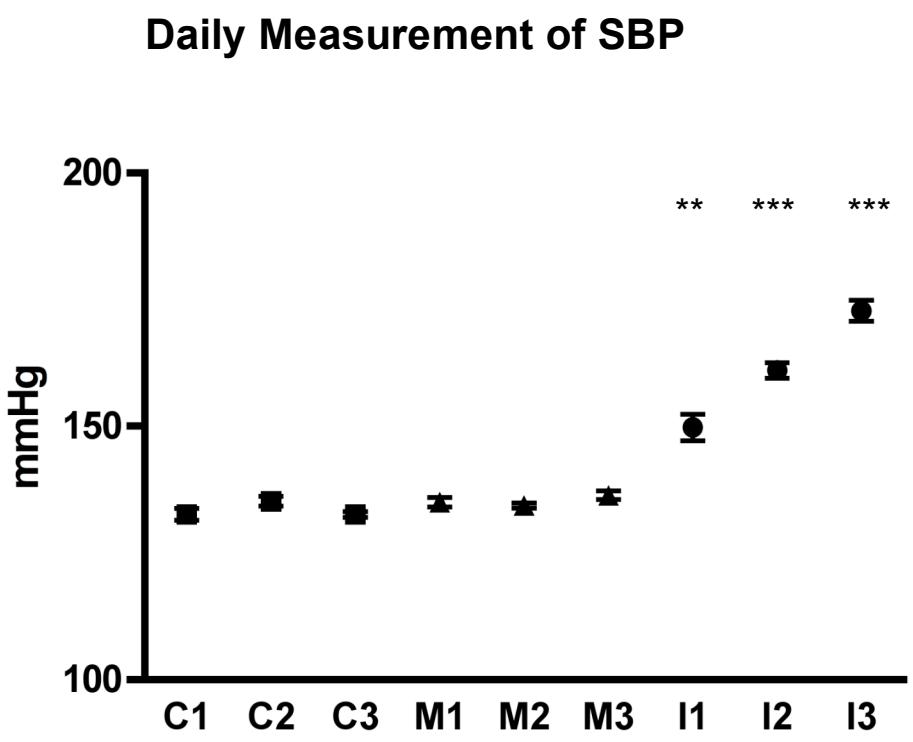


Figure 4-17: (A) Daily measurement of Systolic Blood Pressure by tail cuff plethysmography (N=6). C1 and C3 represent the control period without any induction. M1 to M3 represent the SBP after minipump implantation and before any induction. I1 to I3 represent the induction from day 1 to 3. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. **=$p<0.01$ and ***=$p<0.001$ vs mean of control period readings.

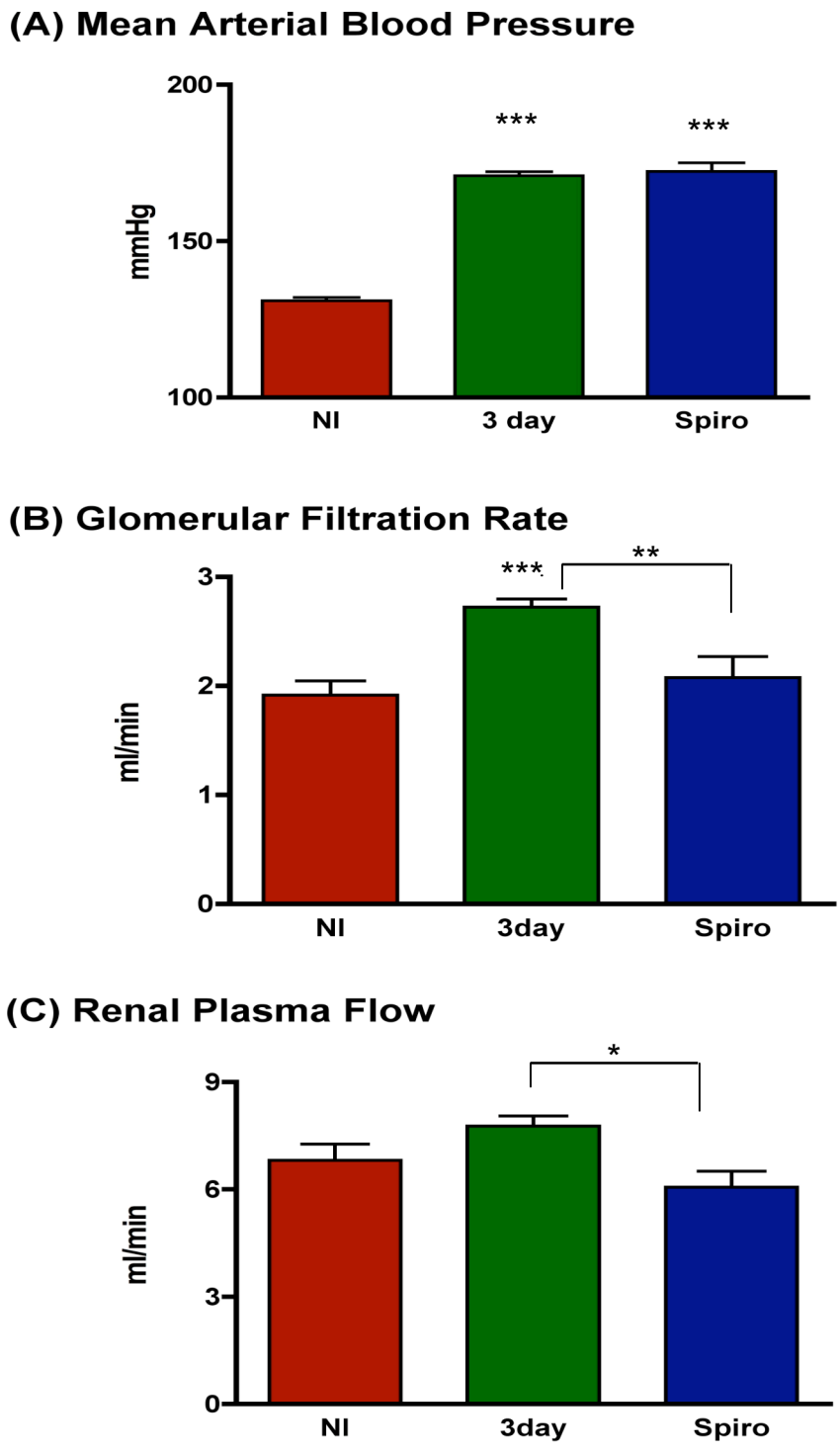


Figure 4-18: (A) MABP, (B) GFR, and (C) RPF of non-induced (NI n=8) 3-day induced (3day, n=9), and MR blockade 3-day induced (Spiro, n=6) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$, **=$p<0.01$ and ***=$p<0.001$ vs NI group.

4.4.4 Effect on renal sodium handling

Urinary excretion of sodium, ENaC mediated sodium transport and NCC mediated sodium transport from non-induced, 3-day induced and MR blockade 3-day induced groups are summarised in Figure 4-19. MR blockade had no significant effect on urinary sodium excretion, with values in the MR blockade 3-day induced group being similar to those of the 3-day induced group. However urinary excretion of sodium was significantly lower in the MR blockade 3-day induced group than in the non-induced group. Moreover MR blockade did not have any effect on sodium retention mediated by either ENaC or NCC.

4.4.5 Effect on urinary albumin excretion

Figure 4-20 shows the urinary albumin excretion of non-induced, 3-day induced and MR blockaded 3-day induced groups. Urinary albumin excretion was increased nearly two fold following 3 days of induction. Chronic inhibition of MR by spironolactone significantly reduced urinary albumin excretion in non-induced rats and prevented the development of microalbuminuria during transgene induction.

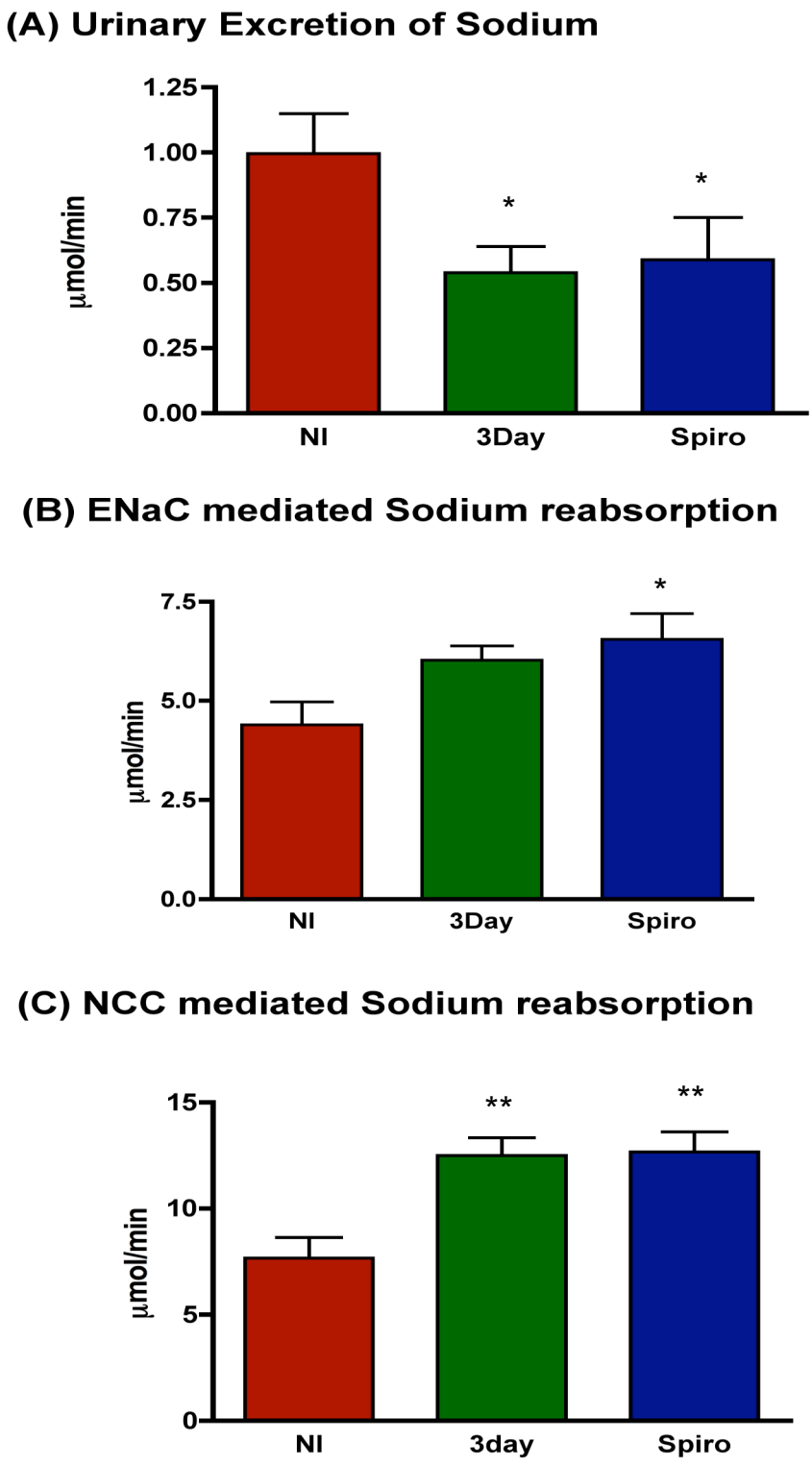


Figure 4-19: (A) Urinary Excretion of sodium, (B) ENaC mediated sodium reabsorption and (C) NCC mediated sodium reabsorption of non-induced (NI, n=8), 3-day induced (3day, n=9) and MR blockade 3-day induced (Spiro, n=6) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and **=$p<0.01$ vs NI group.

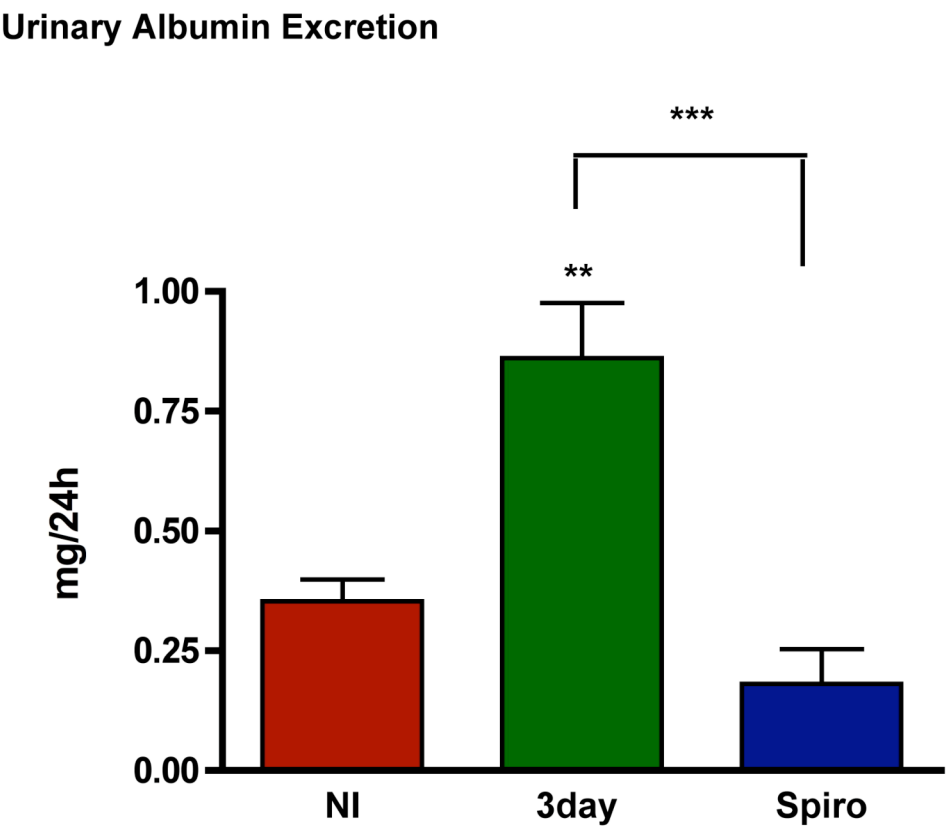


Figure 4-20: Urinary Albumin Excretion of non-induced (NI, n=8), 3-day induced (3day, n=9) and MR blockade 3-day induced (Spiro, n=6) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. **=$p<0.01$ and ***=$p<0.001$ vs NI group.

4.5 Effect of AT_1 receptor blockade on the development of ANGII-dependent hypertension

At the cellular level, the action of ANGII is mediated by two G protein coupled receptors, AT_1 and AT_2. ANGII exerts most of its physiological actions through the AT_1 receptor. Although it has not been measured in the current study, several studies have shown that both plasma and renal ANG II levels were increased in the Cyp1a1-mRen2.F transgenic rat following induction (Kantachuvesiri *et al*., 2001, Mitchel *et al*., 2006). Chronic administration of an AT_1 receptor antagonist prevented the hypertensive phenotype in Cyp1a1-mRen2.F transgenic rats induced with I3C (Mitchel *et al*., 2006, Vanourkova *et al*., 2006). The main objective of the present study was to investigate the role of ANGII in the development of Cyp1a1-mRen2 transgene induced hypertension. Additionally its role in the altered renal sodium handling was assessed. AT_1 receptors were blocked prior to and during induction by administration of losartan in the drinking water.

4.5.1 Effect on blood pressure

To assess the role of ANGII in the development of hypertension, daily SBP was measured by tail cuff plethysmography during the experimental regime (for detail see Figure 2.5). Figure 4-21A shows that AT_1 receptor blockade had no effect on blood pressure before induction of transgenic rats. Induction resulted in a significant increase in blood pressure. However, as shown in figure 4-21B the increase was blunted in the losartan treated 3-day induced group compared to 3-day induced group. On day 3 of induction rats were anaesthetized for MABP measurement and

analysis of renal function. Losartan significantly reduced the MABP relative to the 3-day induced group (Figure 4-22A). However blood pressure in losartan treated 3-day induced group was still significantly higher than that of non-induced group. This indicates that AT_1 receptors blockade blunted the hypertensive phenotype partially.

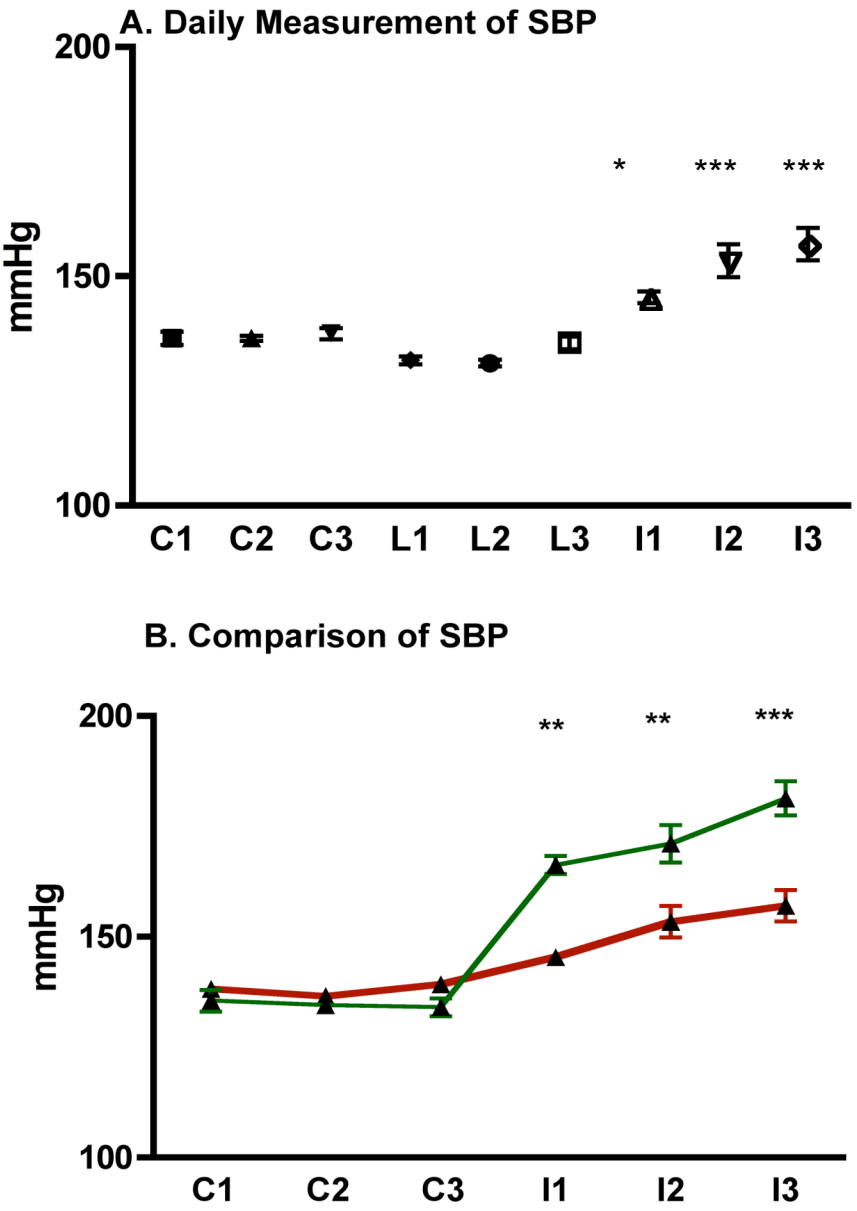


Figure 4-21: (A) Daily measurement of Systolic Blood Pressure by tail cuff plethysmography (n=6). C1 to C3 represent the control period, L1 to L3 represent the time period when AT_1 receptor was blocked by losartan before any induction. I1 to I3 represent the induction period from day 1 to 3 when AT_1 receptor was blocked by losartan. Data are mean±SE and statistical comparisons were made by repeated measures ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and ***=$p<0.001$ vs mean of control period readings.

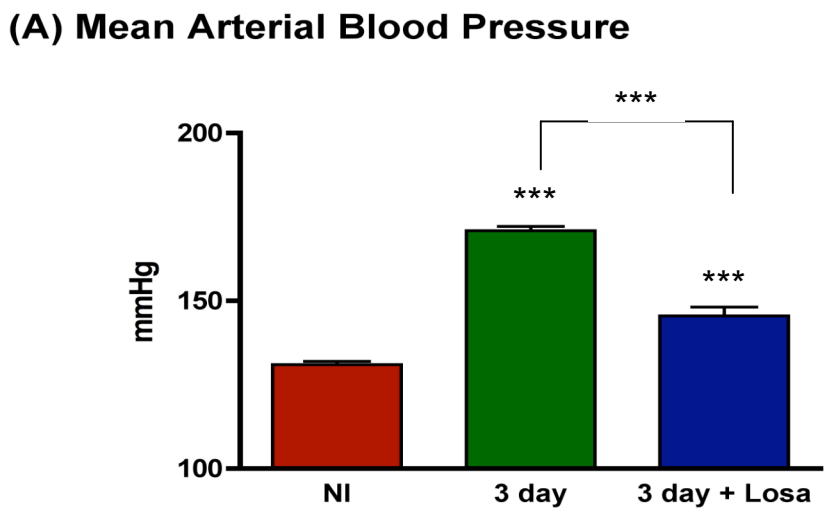
(B) Comparison of Systolic Blood Pressure by tail cuff plethysmography between 3-day induced group (green line) and AT_1 receptor blocked 3-day induced group (Red line). C1 to C3 represent the control period without any induction and I1 to I3 represent the induction period from day 1 to 3. Data are mean±SE and statistical comparisons were made by two way ANOVA with Tukey's post hoc analysis. **=$p<0.01$ and ***=$p<0.001$.

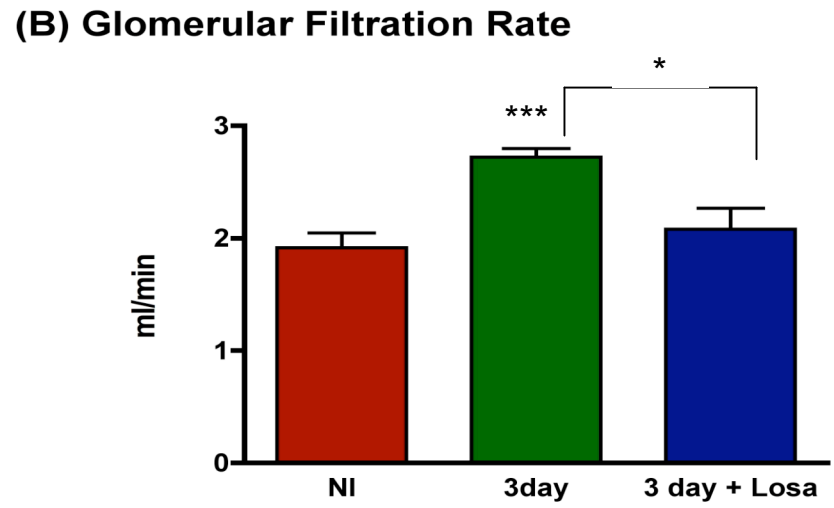
4.5.2 Effect on renal haemodynamics

Figure 4-22 shows the GFR and RPF of non-induced, 3-day induced and AT_1 receptor blockade 3-day induced group. The GFR of AT_1 receptor blocked 3-day induced group was comparable with non-induced control group. Moreover it was significantly reduced compared to the 3-day induced rats. Thus transgene induced elevation of GFR was normalized by AT_1 receptor blockade. However no significant change in RPF was observed due to AT_1 receptor blockade.

4.5.3 Effect on renal sodium handling

Urinary excretion of sodium, ENaC mediated sodium transportation and NCC mediated sodium transportation from non-induced, 3-day induced and AT_1 receptor blockade 3-day induced group are summarised in Figure 4-23. As shown in figure 4-23A, AT_1 receptor blockade prevented the increased sodium reabsorption that occurred following 3 days of induction. ENaC mediated sodium reabsorption was not different between the groups (Figure 4-23.B). However AT_1 receptor blockade prevented the activation of NCC mediated by Cyp1a1-mRen2 transgene induction (Figure 4-23C).





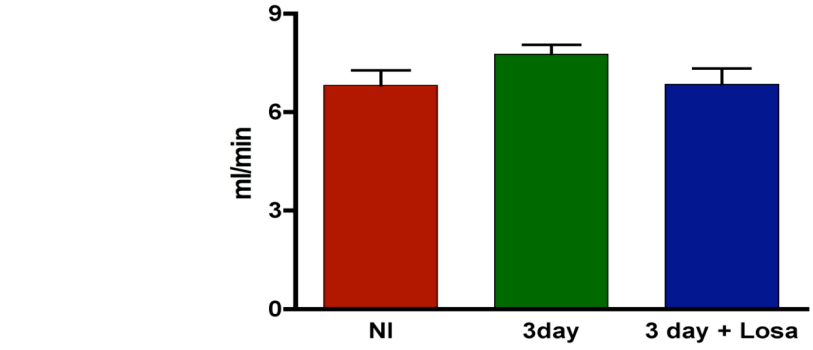


Figure 4-22: (A) MABP, (B) GFR, and (C) RPF of non-induced (NI, n=8) 3-day induced (3day, n=9), and AT_1 receptor blocked 3-day induced (3day+Losa, n=6) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and ***=$p<0.001$ vs NI group.

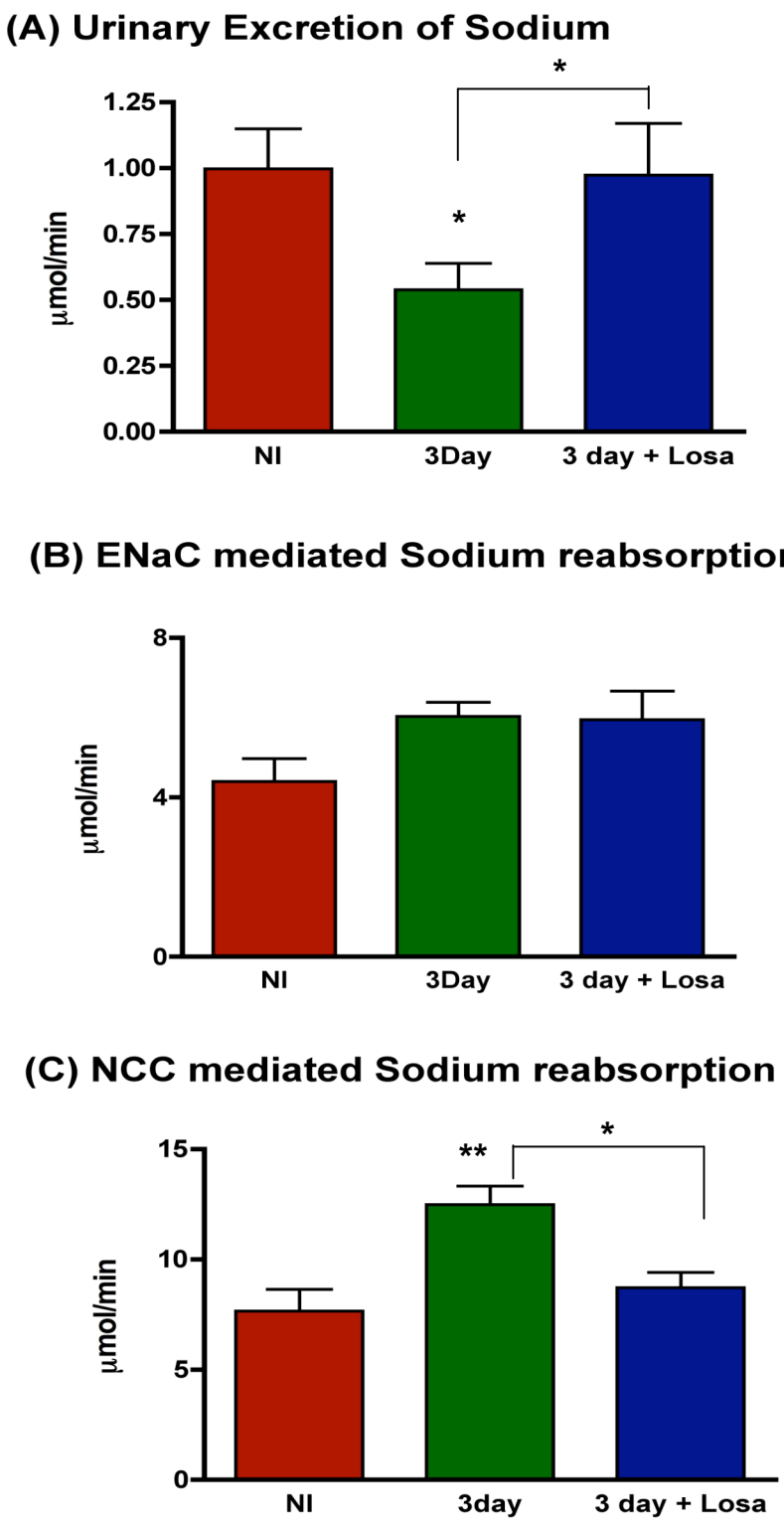


Figure 4-23: (A) Urinary Excretion of Sodium, (B) ENaC mediated Sodium reabsorption, and (C) NCC mediated Sdoium reabsorption of non-induced (NI, n=8) 3-day induced (3day, n=9), and AT_1 receptor blocked 3-day induced (3day+Losa, n=6) group. Data are mean±SE and statistical comparisons were made by one way ANOVA with Tukey's post hoc analysis. *=$p<0.05$ and **=$p<0.01$ vs NI.

Chapter 5

Discussion

Hypertension is an important public health challenge as it is a major risk factor for cardiovascular and renal morbidity and mortality (Whelton, 1994). Worldwide, more than 7 million deaths are caused by hypertension every year and approximately one in four adults are affected by hypertension or its consequences (Kearney, 2005). Better management of hypertension can reduce the prevalence of cardiovascular disease but with the current poor hypertension control rates worldwide, more individuals are being referred for resistant or refractory hypertension (Chobanian, 2009). It is likely that the treatment of hypertension would be improved with a better understanding of the underlying causes. It is well known that activation of the renin–angiotensin system occurs in a significant proportion of hypertensive patients hypertension and predisposes patients to progressive chronic kidney disease. Even in patients with essential hypertension who have no obvious signs of RAAS activation, drugs that target the system can be therapeutically effective. Experimentally, several approaches have documented the critical role of angiotensin II, the active component of the RAAS, in the development of hypertension. It is well established, for example, that chronic administration of sub-pressor doses of angiotensin II cause a slowly developing increase in arterial blood pressure (Simon *et al*., 1995) in both man and experimental animals. In the Goldblatt model of renal vascular hypertension, excessive production of renin drives the production of angiotensin II which in turn causes the development of hypertension. Each of these models displays altered renal function (Navar *et al*., 1998, Wang *et al.*, 2000) and a sensitivity of blood pressure to dietary salt has also been reported (Ando *et al*., 1990). More recently, cross

transplantation studies were used to identify altered renal function as a key component of angiotensin II-dependent hypertension (Crowley *et al*., 2006).

In the current thesis, the Cyp1a1-mRen2.F transgenic rat was used to model ANG II-dependent hypertension. In this model, the production of renin by the mouse Ren2 transgene causes activation of the RAAS and an increase in blood pressure. Transcription of the mRen2 transgene is controlled by the inducible Cyp1a1 promoter: in the current experiments an induction protocol was used that typically drives the hypertension from a stable, "essential" status to a malignant phase within 7-10 days (Liu *et al*., 2009). It was hypothesized that abnormal renal function contributed to the development of hypertension and to the transition from the essential to malignant phase. It was found that the hypertension was sodium-dependent and associated with the enhanced tubular sodium reabsorption. The effect was localized to the distal nephron, specifically to AT_1 receptor-mediated activation of NCC.

5.1 Hypertension develops in a time-dependent manner

The Cyp1a1 inducible promoter offers an experimentally tractable system for induction of hypertension in the Cyp1a1-mRen2 transgenic rat. In earlier studies, it has been shown that the transgene can be induced in a dose-dependent manner. Different levels of transgene induction cause different levels of hypertension with varying severity (Mitchell *et al*., 2006). In particular, a higher dose of I3C (0.3% w/w) by dietary supplement causes a severe hypertension within a week. Conversely a lower dose of I3C (0.15% w/w) for 2 weeks induces a slow developing mild

hypertension which is comparable with the phenotypes of ANG II-infused hypertensive rats, 2K1C Goldblatt hypertensive rats and TGR(mRen2)27 transgenic rats (Von Thun *et al*., 1994; Guan *et al* 1992; Zou *et al*., 1998; Mitchell *et al*., 1997).

The current study demonstrated that induction of Cyp1a1-mRen2.F transgenic rats with I3C for 1, 3 and 7-days resulted in a time-dependent increase in blood pressure. In non-transgenic rats, I3C did not alter blood pressure. This suggested that the effects of I3C on blood pressure in transgenic rats were not from the result of a non-specific action of I3C rather due specifically to activation of the Cyp1a1-mRen2 transgene. Indeed, transgene induction for up to 3-days resulted in a comparatively mild hypertension. A more severe hypertension was observed following 7-days induction at which point the blood pressure had increased ~70mmHg over baseline. Using this dosing regime, the acceleration of blood pressure occurs typically after 6-7 days of induction (Liu *et al*., 2009) on the Fischer 344 background, and the transition from essential to malignant phase of hypertension. At this point following 7-days of induction, characteristic manifestations of malignant hypertension in rodents, such as a pronounced body weight loss, polyuria, lethargy and a hunched posture (Whitworth *et al*., 1994, Whitworth *et al*., 1995) were observed in some of the rats. Thus the Cyp1a1-mRen2.F transgenic rat model allows us to induce the transgene expression and subsequently hypertension in a controlled manner. Specifically, in this study the induction of transgene at different time points facilitated the opportunity to study the renal changes that occurred during the development of hypertension.

5.2 Measurement of blood pressure in conscious and anaesthetized rats

Accurate measurement of blood pressure is essential in the study of hypertension. The determined values of blood pressure, their reliability and appropriate interpretation are highly influenced by the method selected. Thus, a particular technique for measuring BP may be well suited for one type of study but less useful for another. In the present study the effect of transgene induction on blood pressure was measured using two different methods. Tail cuff plethysmography was used to measure blood pressure in conscious rats and in anaesthetized rats MABP was determined by direct cannulation of a carotid artery. Both of these methods have advantages and disadvantages.

5.2.1 Measurement of blood pressure by tail cuff plethysmography

Tail cuff plethysmography is the most commonly used indirect method for blood pressure measurement in animals. A variety of blood flow sensors have been devised for sensing the pressure at which blood flow changes during manipulation of cuff occlusion pressure (Kurtz *et al*., 2005). The main advantage of this indirect method is that it is noninvasive and does not require surgery. Additionally it facilitates the use of same animal to obtain repeated measurements of blood pressure and can be performed on conscious animals without the interference of anaesthetic (Krege *et al*., 1995; Meneton *et al*., 2000; Van Vliet *et al*., 2000). The major drawback of the tail cuff method is that it requires the physical restraint of the animal. In addition the

animal must be preheated to increase blood flow through the tail vein to ensure that tail blood flow is sufficient for measurement. The substantial amount of stress imposed by the restraint and preheating is known to affect BP, heart rate and stress hormones (Bunag & Butterfield, 1982; Gross & Luft, 2003; Popovic, 1988; Irvine *et al*., 1997). The restraint stress can be minimized by taking a great care to train the animals for at least 5-7 days prior to experiment. This period of training allows the animals to acclimatize to the heating chamber and restraint (Gross & Luft, 2003).

In the current study animals were acclimatized for at least 5 days to minimize the impact of stress and to lessen motion artifacts during the measurement procedure. Blood pressure measurement data were taken only when a consistent blood pressure reading was obtained on two consecutive days. In addition to proper training, a cover was placed over the animals, the equipment kept free from foreign scent and urine odor was used to reduce stress and improve measurement reliability. However due to the limitation of the tail cuff method, any change observed in tail cuff blood pressure may not be attributed to the experimental treatments only. A portion of blood pressure changes may be an artifact caused by the animal's stress. Thus in the current study, along with tail cuff measurement, the blood pressure was also measured by direct cannulation of a carotid artery.

5.2.2 Direct measurement of blood pressure by cannulation

Direct recording of BP using fluid-filled catheters is the oldest and most widely used method for the direct measurement of arterial pressure. This technique is very versatile and can be used effectively for acute studies in anaesthetized animals or for

long-term, continuous monitoring of arterial pressure in conscious animals (Van Vliet *et al*., 2000). Long-term recording requires a special arrangement of a swivel and tether system, additionally care must be taken to ensure the continued patency of the arterial catheter. Moreover catheter implantation and surgery cause disturbance on blood pressure and heart rate. In the current study direct measurement of blood pressure by this method was only performed in acute settings. As the rats were induced with I3C daily by gastric gavage it was not possible to monitor blood pressure over the long term in a chronic setting, using indwelling carotid arterial cannulae. In the current study acute settings allowed us to perform renal clearance and blood pressure monitoring at the same time. Additionally we were able to examine the effect of amiloride and thiazide administration on blood pressure. The main disadvantage of this system is the effect of anaesthetic and surgical manipulation as discussed below.

In the current experiment direct blood pressure was measured in terminally anaesthetized rats using thiobutabarbital (Inactin) anaesthetic and the following discussion will be limited to that class of drug. Barbiturate anaesthesia typically induces only a modest depression in blood pressure (Walker *et al*., 1983) that is smaller than those produced by other anaesthetic classes such as propofol (Stoelting & Miller, 2007). This makes barbituates an ideal choice for renal physiological studies: the change in blood pressure is usually within the autoregulatory range, consequently renal perfusion pressure is maintained at near normal levels. The decrease in blood pressure is mainly caused by peripheral vasodilation and depressed sympathetic nervous system outflow from the CNS and may be offset, to a degree, by a compensatory tachycardia (Walker *et al*., 1983). Indeed, following surgical

intervention (Walker *et al*., 1983), blood pressure may be comparable or indeed higher than in conscious animals, due to enhanced CNS-mediated effects.

5.2.3 Radio telemetry

This is the most comprehensive and reliable technology to monitor blood pressure in conscious freely moving laboratory animals (Brockway *et al*., 1991; Kramer *et al*., 2001). The technology has been extensively validated and is available for use in all laboratory animals. Implantable radiotelemetry has the advantage of direct and continuous measurement of BP without the need for restraint or tethering devices. The continuous data acquisition facility of radiotelemetry provides the opportunity to measure BP around the clock for extended periods of time. (Kramer *et al*., 2001; Kramer & Kinter, 2003) The main advantage of this method is the large number of measurements that can be obtained from a single animal, which has substantial effect on the precision of the estimates and the number of animals required to estimate BP accurately (Van Vliet *et al*., 2000). Blood pressure measurement using telemetry was beyond the scope of the current study, however, we have previously reported the blood pressure data following transgene induction measured using radio telemetry (Liu *et al*., 2009).

5.2.4 Comparison of blood pressure recorded from conscious and anaesthetized rats

In a previous study from our lab, blood pressure was measured in conscious Cyp1a1-mRen2.F transgenic rats by radio telemetry (Liu *et al*., 2009). Prior to transgene induction, the baseline MABP recorded was approximately 112 mmHg and

following a 7-day induction, had increased to approximately 160 mmHg; at net gain of ~50mmHg. In the current study, the MABP in conscious rats was measured prior to induction and during the induction by tail cuff plethysmography. The baseline MABP recorded in this way was similar to that recorded by telemetry and the rise in BP following a 7-day induction period was ~70mmHg. However the blood pressure rises recorded by tail cuff plethysmography was comparatively higher than that recorded with telemetry and it may be related to the stress associated with the tail cuff plethysmography blood pressure measurement system. Under Inactin anaesthesia, baseline BP was comparatively higher than that recorded in either of the conscious profiles but the increment following 7 days of induction was of a similar magnitude. This apparent pressor effect of Inactin has been reported before and may reflect activation of the SNS by surgical intervention (Walker *et al*., 1983; Vasthare *et al.,* 1988). Although in the current study comparatively higher MABP was recorded in direct measurement of blood pressure by cannulation, we are unable to determine whether this was the result of stress associated with anaesthesia or surgery. In summary, however, the data obtained under terminal anaesthesia are comparable in terms of the size of the hypertensive response to data obtained in conscious rats.

5.3 Natriuretic capacity and sodium retention

Guyton and colleagues hypothesized that the kidney plays a central role in blood pressure regulation and an alteration in renal function or resetting of the pressure-natriuresis relationship is a prerequisite for the development of hypertension (Guyton, 1972). Simply, pressure-natriuresis means that an elevation in perfusion

pressure in the renal artery causes an increase in sodium and water excretion by the kidney. They further hypothesized that the substantial capacity for sodium excretion by the kidney provides a compensatory system of virtually infinite gain to oppose processes, including increases in peripheral vascular resistance, which would tend to increase blood pressure (Guyton, 1972; Guyton *et al*., 1991). Due to impairment in renal excretory function the relationship between renal sodium excretion and renal perfusion pressure is shifted chronically toward higher levels of BP and this impairment precedes the development of hypertension (Aperia *et al*., 1972).

The mechanism of pressure natriuresis is multifactorial and responds differently to acute and chronic increases of blood pressure. When blood pressure is increased acutely, pressure natriuresis appears to be the result of a rapid decrease in proximal tubule salt and water reabsorption. It is mediated by a rapid redistribution of apical proximal tubule NHE3 from the microvilli to intermicrovillar clefts and then endosomal pools, and suppression of basolateral Na,K-ATPase activity (McDonough *et al*., 2003). In chronic settings pressure natriuresis is achieved by inhibition of tubular sodium transport in distal nephron and collecting duct segments as a consequence of changes in the activity of the ENaC and NCC (Navar & Majid, 1996; Navar *et al*., 1986).

It is well established that activation of the RAS markedly suppresses the pressure-natriuresis relationship (Hall *et al*., 1999; Hall *et al*., 1990). Several studies have demonstrated a rightward shift in the pressure-natriuresis relationship in different types of hypertension (Gross *et al*., 1995; Gross *et al*., 1997; Roman & Cowley 1985;

Roman, 1986). In the rat, infusion of ANG II leads to marked impairment of sodium excretion and suppression of the pressure-natriuresis relationship (Wang *et al*., 2000; Van der Mark & Kline, 1994). AT_1 receptor blockade prevented the suppression of pressure-natriuresis and the hypertension, suggesting that pressure-natriuresis modulation by ANG II is primarily mediated by AT_1 receptor (Wang *et al*., 2000). Similarly in transgenic rats harbouring both human renin and human angiotensinogen genes (Dehmel *et al*., 1998) or transgenic rat for mouse renin gene, TGR(mRen2)27 (Gross *et al*., 1994) the renal pressure-natriuresis mechanism is reset towards higher pressure levels and participates in the maintenance of hypertension. A marked attenuation in the pressure-natriuresis relationship has also been identified as a prime contributor for the development of hypertension in the Cyp1a1-mRen2.F transgenic rat model (Erbanova *et al*., 2009). Enhanced tubuloglomerular feedback likely contributes to the inability of the kidney to maintain normal rates of sodium excretion (Mitchell & Mullins, 2005). The pressure-natriuresis relationship is maintained by accurate regulation of renal haemodynamics and tubular reabsorption. Thus derangement of renal haemodynamics and tubular transport by ANG II are responsible for altered pressure-natriuresis.

The pressure natriuresis response can be measured by measuring the natriuresis induced by an acute pressure ramp. This was not performed in the current thesis but nevertheless we have indirect data suggesting impaired renal natriuretic capacity. In the present study it has been observed that the induction of the Cyp1a1-mRen2 transgene did not significantly change either GFR or RPF over the experimental time-course. This is discussed in more detail in section 5.6 below. However this

observation put forward the suggestion that enhanced tubular salt and water reabsorption might contribute importantly to the maintenance of the hypertensive phenotype. Indeed, the current study shows that urinary sodium excretion is markedly altered in Cyp1a1-mRen2.F rats following I3C administration in response to the infusion of a saline load, thus there is a blunted natriuresis compared to non-induced rats. One possibility might be the alteration in filtration fraction by ANG II. This may lead to augmentation in proximal tubular solute and fluid reabsorption (Kline & Liu 1994; Mattson *et al*., 1991; Roman *et al*., 1988; Roman & Kaldunski, 1991) and thereby contribute to an inappropriately augmented tubular reabsorptive capability in the hypertensive rats. Another possibility might be the direct or indirect action of ANG II to stimulate tubular sodium transport activity (Peti-Peterdi *et al*., 2002; Komlosi *et al*., 2003; Burns & Li, 2003; Beutler *et al*., 2008; Barreto-Chavas & Mello-Aries, 1996; Wang & Giebisch, 1996). In the current study fractional sodium excretion was reduced in each of the experimental groups following induction, suggesting that the antinatriuresis was of tubular origin. However no change in filtration fraction was observed following transgene induction Furthermore, the fractional excretion of lithium, an indirect marker for the proximal tubule sodium reabsorption, (Thomsen *et al*., 1969) was elevated after 1- and 3-days of hypertension, indicating diminished proximal tubular reabsorption (Table 3.2; ANOVA $P<0.001$). However, there was evidence for enhanced proximal reabsorption in the 7-day induced group, but the C_{Na}/C_{Li} ratio, a marker for distal sodium reabsorption, was suppressed throughout the induction regimen (Table 3.2; ANOVA $P<0.05$). Thus all these evidence localizing the antinatriuresis to the distal nephron.

5.4 Balance study

The renal clearance studies clearly indicate that activation of the RAAS is associated with an impaired natriuretic response to a sodium load but this does not necessarily mean that sodium retention plays a key role in the development of the hypertension in this model. A metabolic cage study was therefore performed to examine sodium homeostasis in conscious animals both before and during the induction of the Cyp1a1-mRen2 transgene. We found no evidence of altered daily sodium balance following transgene induction from this experiment. Indeed, there was no evidence at all for expansion of plasma volume, even transiently, during transgene induction. In the current study polyuria was observed in transgenic rats but at same time there was a parallel increase in daily water intake and thus there was no alteration in net water balance. In fact there was a significant linear trend ($P<0.01$) for haematocrit to increase with increasing duration of transgene induction. Although plasma volume was not measured directly, using Evans blue for example (el-Sayed, 1995), increased haematocrit is consistent with a contraction in plasma volume, rather than volume expansion.

It is classically established that sodium retention is followed by water retention, leading to volume expansion. Guyton's hypothesis rests on the fact that this is transient and pressure natiruresis leads to diuresis and restoration of plasma volume (Guyton, 1991). However, recent data challenge this dogma, showing that substantial amounts of sodium could be accumulated without causing water retention via

osmotically inactive sodium storage or an osmotically neutral cation exchange mechanism (Titze, 2009). Water-free sodium retention attenuates the blood pressure by maintaining the ECV close to normal even after increased sodium retention. Thus volume retention and the associated body weight increase do not always parallel sodium retention (Titze *et al*., 2003; Titze *et al*., 2004). This osmotically inactive sodium storage leads to hypertonicity and osmotic stress in the skin interstitium. In response to the osmotic stress subcutaneous tissue macrophages express the transcription factor tonicity enhancer binding protein and induce a local regulatory cascade resulting in the secretion of vascular endothelial growth factor C. Consequently the vascular endothelial growth factor C enhances endothelial nitric oxide synthase expression (Machnik *et al*, 2009). Thus hypertonic skin sodium storage provides a buffering mechanism for hypertension (Machnik *et al*, 2009; Titze & Machnik, 2010). However in the current study no evidence of sodium retention was found from the metabolic cage study. This might be attributed to the significant decrease in food intake following transgene induction and thus animals were not in sodium balance. It is well known that ANG II infusion causes a hypertension that is salt sensitive but several studies suggest that this is not associated with significant sodium retention (Ballew & Fink, 2001). It is possible that hypertension followed by increased sodium retention is not exclusively due to volume expansion rather it enhances the sensitivity of the SNS (Ando *et al*., 1991) and VSM (Simon *et al*, 1998) to ANG II. However in the current study there was no significant increase in plasma sodium concentration following transgene induction.

It has been observed that hypokalaemia induces renal structural changes such as

renal hypertrophy and tubulointerstitial injury (Muehrcke & Rosen, 1964, Riemenschneider & Bohle, 1983). In concert, hypokalaemia also causes an alteration in vasoactive mediators which together induce salt sensitivity hypertension (Suga *et al*., 2001). In the current study there was a linear trend toward hypokalaemia, despite which fractional potassium excretion remained robust suggesting a possible role of potassium in the pathogenesis of hypertension in this transgenic rat model. A further study is required to address this issue.

Daily measurements of body weight during the metabolic cage study clearly demonstrate that the development of hypertension was accompanied by a significant body weight loss. Previously it has been reported that the occurrence of a severe form of hypertension with the higher dose of I3C is associated with pronounced body weight loss (Kantachuvesiri *et al*., 2001; Mitchell & Mullins, 2005; Opay *et al*., 2006; Ortiz *et al*., 2007a; Patterson *et al*., 2007; Vanourkova *et al*., 2006) but this trend was not been observed in mild hypertension induced by the lower dose of I3C (Mitchell, 2006). However the rapid body weight loss is not a non-specific effect of I3C as non-transgenic rats did not exhibit any sign of weight loss following I3C administration. This body weight loss can be well explained by a decrease in daily food intake following induction. During the metabolic cage study the daily food intake was found to be decreased after 3 days of induction and this decrease resulted in a continuous reduction of body weight throughout the induction regime. Conversely, in other studies (Ortiz *et al*, 2007; Vanourkova *et al*., 2006) the food intake was not reduced significantly following induction. Rather, those studies reported the accentuated pressure diuresis or ANG II mediated increased catabolism of fat and/or lean tissue as the cause for loss of body weight. In the current study

polyuria was observed in transgenic rats but at same time there was a parallel increase in daily water intake and thus there was no alteration in net water balance. Thus the observed polyuria can almost be accounted for by the polydipsia indicating that the rats are not losing bodyweight as a consequence of urinary water loss. The observed discrepancy in daily food intake might be attributed to the route of administration of I3C. In the current study I3C administration was carried out through gastric gavage but in other studies I3C was administrated as dietary supplementation. The administration of I3C by gastric gavage induced hypertension more rapidly compared to dietary administration which leads to a significant reduction in daily food intake.

5.5 Sodium dependence of the hypertensive response

Although increased renal sodium reabsorption was associated with the development of hypertension, absolute sodium retention was not found. To investigate the role of sodium in the development of hypertension, a study was performed in which rats were maintained on a diet that was essentially sodium-free before and during induction of the Cyp1a1-mRen2 transgene. Under dietary sodium restriction, transgene induction still caused a substantial increase in blood pressure over baseline but this was partially blunted compared to rats maintained on a normal sodium diet. Importantly, on a low sodium diet, no symptoms of malignant hypertension were observed, which might be due to the hypotensive effect of this regimen. These findings suggest that the development of hypertension in this model is salt dependent. In accordance with this observation, it has been reported that in increased

ANG II levels or an impaired response of ANG II production to dietary salt change is associated with the salt-sensitive component of hypertension in different hypertensive rat models such as spontaneously hypertensive rats, ANG II-infused rats and TGR(mRen2)27 rats (Hodge *et al*., 2002; Huskova *et al*., 2007; Osborn *et al*., 2003; Wang *et al*., 2000).

The Cyp1a1-mRen2.F transgenic rat model exhibits salt-dependency in the development of hypertension. Very recently a detailed study on the effect of dietary sodium on the development of hypertension in Cyp1a1-mRen2.F transgenic rats showed that a low salt diet containing 0.01% sodium substantially attenuated the development of hypertension and a high salt diet containing 8% sodium potentiated the course of hypertension (Husková *et al*., 2010). However high salt diets containing 2% and 4% sodium respectively did not have any incremental effect on the development of hypertension. It has also been reported that TGR(mRen2)27 showed an attenuated development of hypertension when fed on low salt diet (Husková *et al*., 2007). Moreover, the pressor response to an acute dose of ANG II was amplified in rats receiving a higher sodium diet compared to rats that received a lower sodium diet, suggesting that the development of ANG II-dependent hypertension is salt dependent.

5.6 Effect of transgene induction on renal haemodynamics

In the current study the possible role of renal haemodynamics was assessed in the development of hypertension. Although the induction of the transgene markedly

increased MABP from the first day of induction, neither GFR nor RPF were affected in a major way. The maintenance of GFR and RPF in a normal range despite the increased MABP suggests a marked elevation of preglomerular vascular resistance in hypertensive Cyp1a1-mRen2.F transgenic rats. Indeed, the renal vascular resistance was found to be elevated after 7 days of induction. It has been reported that the direct action of ANG II on the preglomerular vasculature or the autoregulatory response allows maintenance of normal renal haemodynamic function despite elevated blood pressure (Baber *et al*., 2003; Mitchell & Navar, 1988; Mitchell & Navar, 1995). However several studies have reported a marked suppression of renal haemodynamics following transgene induction in the transgenic rat model (Mitchell & Mullins, 2005; Mitchel *et al*., 2006). Conversely there is also evidence that renal haemodynamics remained unchanged following induction (Opay *et al.,* 2006; Patterson *et al.,* 2005). Furthermore, it has been shown that COX-2-derived vasodilatory metabolites play an important role in the maintenance of renal haemodynamics (Opay *et al.,* 2006) and reduced bioactivity of nitric oxide due to increased superoxide contributes to the elevation of renal vascular resistance (Patterson *et al.,* 2005). Regardless of the mechanism the current study showed that hypertensive Cyp1a1-mRen2.F rats exhibit markedly increased RVR and the pre-glomerular vasculature prevents the transmission of the systemic hypertension to the glomerular capillaries at least up to 7 days of induction.

5.7 Mechanism of enhanced sodium reabsorption

Although the bulk of the glomerular filtrate is reabsorbed in the proximal tubule, sodium reabsorption in the distal nephron segment is responsible for fine-tuning of

sodium excretion (Peti-Peterdi *et al*., 2002; Mullins *et al*., 2006). Several studies have demonstrated the modulatory role of ANG II on distal tubular sodium reabsorption (Beutler *et al*., 2003, Navar *et al*., 2006; Peti-Peterdi *et al*., 2002; Sandberg *et al*., 2007; Wang & Giebisch, 1996; Wang *et al*., 2003). In the distal nephron ANG II directly stimulates the Na^+/H^+ exchanger, NaCl cotransporter (NCC) and amiloride-sensitive sodium channel (ENaC). In particular ANG II enhances sodium reabsorption by stimulating Na^+/H^+ exchanger and the amiloride-sensitive sodium channel (ENaC) in the distal tubule and cortical collecting duct (Barreto-Chaves *et al*., 1996; Schlatter *et al*., 1995; Wang and Giebisch, 1996). It has been shown that ANG II directly increases ENaC activity in isolated perfused rabbit cortical collecting ducts (Peti-Peterdi *et al*., 2002). In AT_{1a} receptor knockout mice, a marked decrease in NCC and αENaC subunit have been reported in response to activated RAAS. This suggests an AT_1 receptor mediated regulatory role of ANG II to these transporter and hence sodium handling (Brooks *et al*., 2002).

In addition to direct effects, ANG II also regulates indirectly the function of distal tubular sodium reabsorption: ANG II stimulates the zona glomerulosa of the adrenal cortex to produce the sodium-retaining hormone, aldosterone, which enhances sodium transport in the cortical collecting duct and distal convoluted tubule. This enhancement is mainly mediated via an effect on ENaC. Aldosterone increases the number of functional ENaC on the cell surface and also increases the channel open probability (Garty & Palmer, 1997). Moreover aldosterone also regulates sodium reabsorption in the distal convoluted tubule by regulating NCC expression. Aldosterone infusion in adrenalectomized rats increased the abundance of NCC

expression. Conversely spironolactone administration in salt restricted rats decreased the abundance of NCC (Neilsen *et al*., 2002).

In the current study, the mechanisms responsible for the observed enhancement of renal sodium reabsorption in the distal tubule were measured using acutely administered blockers of ENaC and NCC. Our data indicate that increased NCC activity is responsible for the enhanced renal sodium retention as ENaC mediated sodium reabsorption was largely unchanged during Cyp1a1-mRen2 transgene induction. Data from the current study clearly show a progressive stimulation of NCC mediated sodium reabsorption during transgene induction. After 7-days of induction the NCC activity was approximately two-fold increased. The increased activity of NCC following induction was also supported by the Western blot analysis. An increase in total kidney NCC abundance was observed due to transgene induction.

To further investigate the role of NCC mediated sodium reabsorption in the development of ANG II dependent hypertension, NCC was chronically inhibited before and during transgene induction. The chronic inhibition of NCC during transgene induction significantly blunted the hypertensive phenotype but did not prevent it completely. These data suggest that increased sodium retention mediated by NCC plays a key role in the development of ANG II-dependent hypertension in this transgenic rat model.

The importance of NCC in the regulation blood pressure has been widely studied. Gene mutation or alteration in the expression of NCC leads to an alteration in blood pressure. Loss-of-function mutations of NCC have been linked to Gitelman's

syndrome, which is characterized by salt wasting, mild hypotension, hypokalaemia, and secondary hyperaldosteronism (Cruze *et al*.,2001; Melander *et al*., 2000). Conversely Gordon's syndrome, another autosomal dominant disorder linked to the increased activity of NCC, which is characterized by hypertension and hyperkalaemia. (Yang *et al*., 2005). Recently it has been shown that acute hypertension provoked the redistribution of NCC from apical to sub-apical cytoplasmic vesicles. However this redistribution is secondary to a reduction in ANG II accompanied by hypertension since clamping of ANG II to a non-pressor level prevented the NCC redistribution (Lee *et al*., 2009). These data suggest the important role of NCC in the regulation of sodium excretion.

5.8 Activation of NCC by phosphorylation

In the current study a transient increase in the abundance of NCC was observed following induction. However it is well established that NCC mediated sodium retention could also be regulated by changing the number or activity of NCC in the apical membrane. It has been shown that apical abundance of NCC can be regulated by ANG II directly and also indirectly by aldosterone (Sandberg *et al*., 2006, Sandberg *et al*., 2007). Acute trafficking of NCC from the sub-apical membrane to the apical plasma membrane was observed following ANG II infusion (Sandberg *et al*., 2007). Conversely, the ACE inhibitor captopril reversed the trafficking (Sandberg *et al*., 2007). ANG II signaling increases NCC activity by abrogating the inhibitory effect of WNK4. This elimination of WNK4 mediated inhibition lead to enhanced phosphorylation of NCC by SPAK/ORS kinases. This phosphorylation

enhanced the trafficking of NCC to the plasma membrane and increased its activity (San-Cristobal *et al*., 2009). Specifically, phosphorylation of two threonine residues T53 and T58 is crucial for NCC activity (Richardson *et al*., 2008).

Based on these data it was hypothesized that not only total protein expression but also enhanced phosphorylation and the subsequent increased apical expression might be responsible for activity. To address this issue, the effect of transgene induction on phosphorylation of the key residue was also analyzed using an antibody specific for phosphorylation at T53 of NCC. However no difference in phosphorylation was observed. Based on this finding we cannot rule out that there was no increase in phophorylation of NCC. SPAK and OSR1 phosphorylate and activate SLC12 family ion co-transporters that includes NCC, NKCC1 and NKCC2 (Vitari *et al*., 2006). Evidence suggests that SPAK and OSR1 directly phosphorylate several threonine residues in a conserved motif R-F-X-[VI] of these co-transporters (Richardson *et al*., 2008). Thus the phospho-antibody not only binds specifically with phosphorylated NCC but also binds with phosphorylated NKCC1 and NKCC2 (personal communication with Professor Dario Alessi, University of Dundee). A further co-immunoprecipitation experiment is required to address this problem.

5.9 Role of ENaC in the development of hypertension

There is ample evidence that ENaC mediated sodium retention plays a major role in salt and water homeostasis in the body and blood pressure regulation (Rossier *et al*., 2002; Garty & Palmer, 1997). Genetically occurring ENaC mutations which lead to

increased or decreased ENaC activity, such as Liddle's syndrome (Liddle *et al*., 1963) and type I pseudohypoaldosteronism (PHA-I) (Chang *et al*., 1996) also imply its physiological importance in blood pressure regulation. However, in the current study pharmacological intervention of ENaC by amiloride did not find any contribution of ENaC mediated sodium transport to the development of hypertension in this model. One possible explanation may be the increased sodium reabsorption along the DCT by the over activation of the upstream sodium transporter NCC, which may limit the Na^+ load that reaches ENaC. Consistent with this explanation, enhanced ENaC mediated sodium reabsorption was observed when NCC was blocked chronically. Further study is necessary to address this possibility.

5.9.1 Western blot analysis for ENaC

ENaC is a hetero-oligomer consisting of three subunits α, β and γ. Among the three subunits, the α-subunit is rate limiting for assembly of mature ENaC complexes essential for normal function of the channel (Beutler *et al*., 2003; May *et al*., 1997). In the current study the expression of the αENaC subunit was measured by Western blot analysis. A transient increase in abundance of αENaC expression was only found following 1 day of induction and this enhancement was not sustained after either 3-day or 7-day induction. Thus Western blot analysis supports the findings from pharmacological intervention of ENaC by amiloride that ENaC mediated sodium reabsorption do not play any contributory role in enhanced sodium retention. However the activity of ENaC does not only depend on the total expression, so based on Western blot analysis alone we cannot rule out the possible contributory role of

ENaC in the development hypertension in this model. Rather there might be an alteration in ENaC trafficking to the apical plasma membrane, ubiquitination, phosphorylation or proteolytic processing which may increase the activity of ENaC without increasing total expression (Bhalla & Hallows, 2008). It has been reported in several studies that activity of ENaC is largely regulated by the kinase SGK1 (Chen *et al*., 1999, Pearce *et al*., 2003) and ubiquitin protein Nedd4-2 (Snyder *et al*., 2002). SGK1 stimulates ENaC activity by direct phosphorylation of a serine residue. It also regulates ENaC through the phosphorylation of the ubiquitin protein Nedd4-2. SGK1 mediated phosphorylation inhibits the binding affinity of Nedd4-2 to the target protein and thus reduces the ubiquitination of ENaC. This causes an increase the abundance of ENaC channel protein in the cell membrane. (Alvarez-de-la-Rosa *et al*., 1999; Debonneville *et al*., 2001). Due to the pivotal role of Nedd4-2 and SGK1 in the regulation of ENaC activity, the abundance the SGK1 and Nedd4-2 was also measured in the current study. However no difference was observed in the total expression. It might be possible that inactivation of Nedd4-2 by SGK1 mediated phosphorylation regulates ENaC activity rather than by a decreas in Nedd4-2 total expression. Again phosphorylation is essential for SGK1 to become functional (Biondi *et al*., 2001; Kobayashi *et al*., 1999). This suggests that a further study is required to analyse the phosphorylation of ENaC and the upstream proteins necessary for its regulation. It would be also interesting to study the effect of induction on trafficking of ENaC from sub-apical space to apical membrane since activity is also regulated by localization.

5.10 Role of AT_1 receptor activation in the development of hypertension

It is well known that inappropriate activation of the RAS plays a major role in the pathogenesis of ANG II -dependent hypertension and ANG II confers most of its action upon binding with AT_1 receptors. In the current study, the AT_1 receptor blocker losartan had no effect on blood pressure in non-induced rats but significantly attenuated the hypertensive response to transgene induction. This finding suggests that the enhanced generation ANG II following the induction of the Cyp1a1-Ren2 transgene causes hypertension in Cyp1a1-Ren2 transgenic rats as a consequence of AT_1 receptor activation. This result confirms the previous finding that treatment with an AT_1 receptor antagonist prevented the development of hypertension in this model (Mitchell *et al*., 2006, Vanourková *et al*., 2006). However a recent study found no effect of chronic administration of AT_1 receptor blockade on systolic blood pressure in this model (Williams *et al*., 2010). Chronic AT_1 receptor blockade also attenuates the development of hypertension in different ANG II -dependent hypertensive rat models such as 2K1C Goldblatt hypertensive rats (Imamura *et al*., 1995), ANG II-infused rats (Zou *et al*., 1996) and TGR(mREN2)27 transgenic rats (Bohm *et al*., 1995). Increased circulating ANG II as a consequence of transgene induction and the augmented intrarenal ANG II lead to the pathogenesis of hypertension in Cyp1a1-mRen2.F transgenic rats (Erbanová *et al*., 2009; Husková *et al*., 2010; Mitchell *et al*., 2006).

In AT_{1a} receptor knockout mice fed on low-salt diet, NCC protein abundance was reported to be reduced compared with wild-type mice (Brooks *et al*., 2002); this implies that NCC expression is regulated directly by ANG II stimulation. A detailed study from Sandberg *et al*. (Sandberg *et al*., 2007) demonstrated the direct role of ANG II in the trafficking of NCC to the apical membrane. Moreover ACE inhibition causes rapid internalization of NCC and thus suppresses NaCl reabsorption in the distal convoluted tubule. Although the collecting duct is the primary site for aldosterone action, aldosterone administration also increased the expression of NCC in the distal convoluted tubule (Kim *et al*., 1998). NCC abundance is increased during dietary NaCl restriction (Masilamani *et al*., 2002) and decreased during a high-salt diet (Sandberg *et al*., 2006; Song *et al*., 2004,) and mineralocorticoid blockade (Nielsen *et al*., 2002). Thus ANG II can directly regulate NCC activity via AT1 receptor activation and also by increased aldosterone synthesis.

In the current study AT_1 receptor inhibition completely normalized the enhanced sodium reabsorption that was observed following transgene induction. In particular losartan therapy completely blunted the enhanced NCC mediated sodium reabsorption. This finding suugests that AT_1 receptor activation by ANG II generated from the induction of the Cyp1a1-mRen2.F transgene is essential for increased NCC activity. In the current study it has already been shown that chronic inhibition of NCC by thiazide completely blocks the NCC activity but only partially attenuates the hypertensive phenotype. Again losartan completely attenuates NCC stimulation, but

only partially blocks hypertension. Thus NCC is important but it is not the only hypertensive mechanism.

5.11 Role of aldosterone in the development of hypertension

It has previously shown that activation of the Cyp1a1-mRen2.F transgene causes a rapid and sustained increase in circulating ANG II concentration. Urinary aldosterone, used as a surrogate for plasma concentration, was found to be increased following induction in the current study and remained sustained throughout the experimental regime. The beneficial effects of losartan, described above, could be attributed to indirect inhibition of aldosterone synthesis. Thus, the contribution of increased aldosterone synthesis in the development of hypertension and its role on tubular sodium reabsorption was assessed. Chronic inhibition of MR by spironolactone did not affect the blood pressure in non-induced rats and was not able to prevent the hypertensive response to transgene induction. This suggests that aldosterone does not play a substantial role in the elevation of blood pressure in Cyp1a1-Ren2.F rats. Several studies have reported that increased aldosterone levels do not contribute significantly to the development of ANG II-dependent forms of hypertension (Kanagy *et al*., 1990; Martinez *et al*., 2002; Ortiz *et al*., 2007; Rocha *et al*., 2000; Rocha *et al*., 2002; Sander *et al*., 1992).

It has been reported that chronic MR antagonism potentiates AT_1 receptor-mediated intrarenal uptake of ANG II. This enhanced intrarenal ANG II may overcome the effect of MR blockade and thus maintain the sustained sodium reabsorption and hypertension (Nielsen *et al*., 2002; Nielsen *et al*., 2007). Alternatively spironolactone

treatment may exert a positive feedback on aldosterone synthesis leading to a further elevation of plasma aldosterone. The elevated aldosterone level might overcome receptor blockade or exerts its effect through the unblocked glucocorticoid receptor (Gaeggeler *et al*., 2005; Ortiz *et al*., 2007b). However MR receptor blockade did not alter either intrarenal ANG II or plasma aldosterone levels in hypertensive Cyp1a1-mRen2.F transgenic rats (Ortiz *et al*., 2007a).

Although in the current study MR blockade failed to alleviate the hypertensive phenotype, it completely prevented the development of microalbuminuria associated with hypertension, suggesting that aldosterone does contribute to the renal damage in Cyp1a1-Ren2.F hypertensive rats but via a mechanism independent of blood pressure. It has been reported that aldosterone induces the production of growth factor by TGF-β signaling pathways which upregulates collagen synthesis, downregulates the release of the extracellular matrix metalloproteinase collagenase, and promotes fibroblast proliferation (Sun *et al*, 2000). Furthermore, aldosterone induces production of reactive oxygen species and expression of inflammatory molecules (Nishiyama *et al*., 2004) and inhibits matrix degradation by enhancing plasminogen activator inhibitor-1 (PAI-1) expression (Brown *et al*., 2000; Ma *et al*., 2006). All of these contribute to the final common pathway of renal fibrosis and cause renal damage. Renal injury has been reported in a variety of hypertensive models, including spontaneously hypertensive rats (SHR), ANG II-infused hypertensive rats, Dahl salt-sensitive rats, DOCA-salt hypertensive rats and TGR[mREN2]27 transgenic rat (Bledsoe *et al*., 2006; Cheng *et al*., 2003; Rodríguez-Iturbe *et al*., 2004). It is well known that selective blockade of aldosterone reduces

proteinuria and nephrosclerosis independent of effects on blood pressure (Epstein *et al.*, 2001). In the malignant hypertensive Cyp1a1-mRen2.F transgenic rat model, renal pathological changes primarily consist of inflammation and cellular proliferation in the cortical vessels and tubulointerstitium (Graciano *et al.*, 2007) and it has been shown that chronic treatment with spironolactone completely alleviated the proteinuria associated with renal injury.

In the current study it has been shown that MR blockade had no significant effect on urinary sodium excretion. Furthermore, MR blockade did not have any effect on sodium retention mediated by NCC. It is well known that aldosterone directly regulates distal tubular sodium transport. However the present findings suggest that AT_1 receptor activation by ANG II play a key role in the regulation of NCC activity and this activation can mask the MR blockade effect. However, further study is required to address this issue.

5.12 Conclusion

In summary, the present findings demonstrate that induction of the Cyp1a1-mRen2 transgene caused a progressive increase in blood pressure in a time dependent manner and this development of angiotensin II-dependent hypertension is mediated by increased tubular sodium reabsorption rather than altered renal blood flow. The enhanced sodium reabsorption in the distal nephron specifically the increased activity of the thiazide-sensitive sodium chloride cotransporter (NCC), is a key hypertensive mechanism in this model. Increased activity of NCC results directly from the actions of ANG II mediated through AT_1 receptor activation. Evidence has been presented in the current study that places NCC in a central position for the development of hypertension in the Cyp1a1-mRen2 transgenic rat. Chronic administration of either hydrochlorothiazide or the AT1 receptor antagonist losartan completely blocked NCC activity and normalized distal tubule sodium reabsorption but only partially attenuated the hypertensive phenotype. Similarly dietary sodium restriction also blunted the hypertensive phenotype partially. These suggest that NCC mediated enhanced sodium reabsorption is important but is not the only hypertensive mechanism in this model. Finally the present study suggests that aldosterone does not contribute to the development of either hypertension or increased NCC activity but contributes to the renal vascular injury in Cyp1a1-Ren2 hypertensive rats.

5.13 Perspectives

The relationship between dietary sodium and the pathogenesis of essential hypertension has been an area of interest and investigation for decades. About 51% of the hypertensive population and 26% of normotensive individuals are salt sensitive (Weinberger, 1996). It has been reported that the prevalence of hypertension is more frequent in salt-sensitive individuals compare to their salt-resistant counterparts (Weinberger & Fineberg, 1991). Furthermore, the incidence of cardiovascular events is 3 fold higher in salt-sensitive hypertensive patients (Moromoto, 1997) and salt-sensitive normotensive individuals have increased mortality independent of blood pressure (Weinberger *et al*., 2001). Transient activation of the RAAS can cause salt-sensitivity to develop which in itself is an important risk factor for cardiovascular disease (Lombardi *et al*., 1999). Transient induction of ANG II-dependent hypertension via activation of the mRen2 transgene induces salt-sensitive hypertension in the Cyp1a1-mRen2 transgenic rat (Howard *et al*., 2005). An increased sodium retention was observed in this salt sensitive hypertension. Enhanced tubular reabsorption was indicated as the key contributor for this impairment as neither GFR nor RPF was altered (Howard *et al*., 2005). In the present study it has been observed that development of ANG II-dependent hypertension is salt-dependent and caused by sustained activation of the thiazide-sensitive cotransporter, NCC. Thus further study is required to investigate the possible relationship between NCC and ANG II induced salt-sensitive hypertension.

References

Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. Biophotonics International. 2004 July; 1(7):36-42.

Aguilera G. Factors controlling steroid biosynthesis in the zona glomerulosa of the adrenal. J Steroid Biochem Mol Biol. 1993 Apr;45(1-3):147-51.

Aguilera G, Kiss A. Regulation of the hypothalmic-pituitary-adrenal axis and vasopressin secretion. Role of angiotensin II. Adv Exp Med Biol. 1996;396:105-12.

Ahmad S, Ward PE. Role of aminopeptidase activity in the regulation of the pressor activity of circulating angiotensins. J Pharmacol Exp Ther. 1990 Feb;252(2):643-50.

Ahmad U, Saleheen D, Bokhari A, Frossard PM. Strong association of a renin intronic dimorphism with essential hypertension. Hypertens Res. 2005 Apr;28(4):339-44.

Al-Dujaili EA, Edwards CR. Development and application of a simple radioimmunoassay for urinary aldosterone. Clin Chim Acta. 1981 Nov;116(3):277-87.

Al-Dujaili EA, Mullins LJ, Bailey MA, Kenyon CJ. Development of a highly sensitive ELISA for aldosterone in mouse urine: validation in physiological and pathophysiological states of aldosterone excess and depletion. Steroids. 2009 Apr-May;74(4-5):456-62.

Allen AM, Zhuo J, Mendelsohn FA. Localization of angiotensin AT1 and AT2 receptors. J Am Soc Nephrol. 1999 Jan;10 Suppl 11:S23-9.

Alvarez de la Rosa D, Zhang P, Náray-Fejes-Tóth A, Fejes-Tóth G, Canessa CM. The serum and glucocorticoid kinase sgk increases the abundance of epithelial sodium channels in the plasma membrane of Xenopus oocytes. J Biol Chem. 1999 Dec ;274(53):37834-9.

Amlal H, LeGoff C, Vernimmen C, Soleimani M, Paillard M, Bichara M. ANG II controls Na(+)-K+(NH4+)-2Cl- cotransport via 20-HETE and PKC in medullary thick ascending limb. Am J Physiol. 1998 Apr;274(4 Pt 1):C1047-56.

Ando K, Sato Y, Fujita T. Salt sensitivity in hypertensive rats with angiotensin II administration. Am J Physiol. 1990 Nov;259(5 Pt 2):R1012-6.

Aperia AC, Broberger CG, Söderlund S. Relationship between renal artery perfusion pressure and tubular sodium reabsorption. Am J Physiol. 1971 May;220(5):1205-12.
Apfeldorf WJ, Rasmussen H. Simultaneous determination of intracellular free calcium and aldosterone production in bovine adrenal zona glomerulosa. Cell Calcium. 1988 Apr;9(2):71-80.

Aguilera G, Marusic ET. Role of the renin angiotensin system on the biosynthesis of aldosterone. Endocrinology. 1971 Dec;89(6):1524-9.

Arendshorst WJ, Brännström K, Ruan X. Actions of angiotensin II on the renal microvasculature. J Am Soc Nephrol. 1999 Jan;10 Suppl 11:S149-61.

Ashton N. Neurological and humoral control of blood pressure. Anaesthesia and Intensive Care Medicine. 2007 June:8(6):221-226.

Averill DB, Diz DI. Angiotensin peptides and baroreflex control of sympathetic outflow: pathways and mechanisms of the medulla oblongata. Brain Res Bull. 2000 Jan;51(2):119-28.

Baber SR, Hyman AL, Kadowitz PJ. Role of COX-1 and -2 in prostanoid generation and modulation of angiotensin II responses. Am J Physiol Heart Circ Physiol. 2003 Dec;285(6):H2399-410.

Bader M, Peters J, Baltatu O, Müller DN, Luft FC, Ganten D. Tissue renin-angiotensin systems: new insights from experimental animal models in hypertension research. J Mol Med. 2001 Apr;79(2-3):76-102.

Bader M, Zhao Y, Sander M, Lee MA, Bachmann J, Böhm M, Djavidani B, Peters J, Mullins JJ, Ganten D. Role of tissue renin in the pathophysiology of hypertension in TGR(mREN2)27 rats. Hypertension. 1992 Jun;19(6 Pt 2):681-6.

Bailey MA, Giebisch G, Abbiati T, Aronson PS, Gawenis LR, Shull GE, Wang T. NHE2-mediated bicarbonate reabsorption in the distal tubule of NHE3 null mice. J Physiol. 2004 Dec;561(Pt 3):765-75.

Baker KM, Booz GW, Dostal DE. Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. Annu Rev Physiol. 1992;54:227-41.

Baker KM, Johns DW, Vaughan ED Jr, Ayers CR, Carey RM. Antihypertensive effects of angiotensin blockade: saralasin versus captopril. Clin Exp Hypertens. 1980;2(6):947-54.

Ballew JR, Fink GD. Characterization of the antihypertensive effect of a thiazide diuretic in angiotensin II-induced hypertension. J Hypertens. 2001 Sep;19(9):1601-6.

Barker PM, Nguyen MS, Gatzy JT, Grubb B, Norman H, Hummler E, Rossier B, Boucher RC, Koller B. Role of gammaENaC subunit in lung liquid clearance and electrolyte balance in newborn mice. Insights into perinatal adaptation and pseudohypoaldosteronism. J Clin Invest. 1998 Oct;102(8):1634-40.

Barreto-Chaves ML, Mello-Aires M. Effect of luminal angiotensin II and ANP on early and late cortical distal tubule HCO3- reabsorption. Am J Physiol. 1996 Nov;271(5 Pt 2):F977-84.

Beevers G, Lip GY, O'Brien E. ABC of hypertension: The pathophysiology of hypertension. BMJ. 2001 Apr;322(7291):912-6.

Bertaux F, Colledge WH, Smith SE, Evans M, Samani NJ, Miller CC. Normotensive blood pressure in mice with a disrupted renin Ren-1d gene. Transgenic Res. 1997 May;6(3):191-6.

Betancourt-Calle S, Mann-Blakeney RS, Isales CM, Calle RA, Bollinger Bollag W. Angiotensin II priming of aldosterone secretion with agents that enhance Ca(2+) influx. Mol Cell Endocrinol. 2001 May;177(1-2):61-70.

Beutler KT, Masilamani S, Turban S, Nielsen J, Brooks HL, Ageloff S, Fenton RA, Packer RK, Knepper MA. Long-term regulation of ENaC expression in kidney by angiotensin II. Hypertension. 2003 May;41(5):1143-50.

Bhalla V, Hallows KR. Mechanisms of ENaC regulation and clinical implications. J Am Soc Nephrol. 2008 Oct;19(10):1845-54.

Bianchi G, Fox U, Pagetti D, Caravaggi AM, Baer PG, Baldoli E. Mechanisms involved in renal hypertension. Kidney Int Suppl. 1975 Sep:S165-73.

Biondi RM, Kieloch A, Currie RA, Deak M, Alessi DR. The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. EMBO J. 2001 Aug;20(16):4380-90.

Bird IM, Pasquarette MM, Rainey WE, Mason JI. Differential control of 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. J Clin Endocrinol Metab. 1996 Jun;81(6):2171-8.

Blair-West JR, Reid IA, Ganong WF. Stimulation of angiotensinogen release by raised blood angiotensin concentration in the dog. Clin Sci Mol Med. 1974 May;46(5):665-9.

Bledsoe G, Shen B, Yao Y, Zhang JJ, Chao L, Chao J. Reversal of renal fibrosis, inflammation, and glomerular hypertrophy by kallikrein gene delivery. Hum Gene Ther. 2006 May;17(5):545-55.

Böhm M, Lee M, Kreutz R, Kim S, Schinke M, Djavidani B, Wagner J, Kaling M, Wienen W, Bader M, et al. Angiotensin II receptor blockade in TGR(mREN2)27: effects of renin-angiotensin-system gene expression and cardiovascular functions. J Hypertens. 1995 Aug;13(8):891-9.

Bonnardeaux A, Davies E, Jeunemaitre X, Féry I, Charru A, Clauser E, Tiret L, Cambien F, Corvol P, Soubrier F. Angiotensin II type 1 receptor gene polymorphisms in human essential hypertension. Hypertension. 1994 Jul;24(1):63-9

Braam B, Mitchell KD, Koomans HA, Navar LG. Relevance of the tubuloglomerular feedback mechanism in pathophysiology. J Am Soc Nephrol. 1993 Dec;4(6):1257-74.

Braam B, Navar LG, Mitchell KD. Modulation of tubuloglomerular feedback by angiotensin II type 1 receptors during the development of Goldblatt hypertension. Hypertension. 1995 Jun;25(6):1232-7.

Breault L, Lehoux JG, Gallo-Payet N. Angiotensin II receptors in the human adrenal gland. Endocr Res. 1996 Nov;22(4):355-61.

Brenner R, Peréz GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT, Aldrich RW. Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. Nature. 2000 Oct;407(6806):870-6.

Brockway BP, Mills PA, Azar SH. A new method for continuous chronic measurement and recording of blood pressure, heart rate and activity in the rat via radio-telemetry. Clin Exp Hypertens A. 1991;13(5):885-95.

Brooks HL, Allred AJ, Beutler KT, Coffman TM, Knepper MA. Targeted proteomic profiling of renal Na(+) transporter and channel abundances in angiotensin II type 1a receptor knockout mice. Hypertension. 2002 Feb;39(2 Pt 2):470-3.

Brooks HL, Sorensen AM, Terris J, Schultheis PJ, Lorenz JN, Shull GE, Knepper MA. Profiling of renal tubule Na+ transporter abundances in NHE3 and NCC null mice using targeted proteomics. J Physiol. 2001 Feb;530(Pt 3):359-66.

Brown NJ, Nakamura S, Ma L, Nakamura I, Donnert E, Freeman M, Vaughan DE, Fogo AB. Aldosterone modulates plasminogen activator inhibitor-1 and glomerulosclerosis in vivo. Kidney Int. 2000 Sep;58(3):1219-27.

Bunag RD, Butterfield J. Tail-cuff blood pressure measurement without external preheating in awake rats. Hypertension. 1982 Nov-Dec;4(6):898-903.

Burns KD, Li N. The role of angiotensin II-stimulated renal tubular transport in hypertension. Curr Hypertens Rep. 2003 Apr;5(2):165-71.

Burson JM, Aguilera G, Gross KW, Sigmund CD. Differential expression of angiotensin receptor 1A and 1B in mouse. Am J Physiol. 1994 Aug;267(2 Pt 1):E260-7.

Butterworth MB. Regulation of the epithelial sodium channel (ENaC) by membrane trafficking. Biochim Biophys Acta. 2010 Dec;1802(12):1166-77.

Campbell DJ, Habener JF. Angiotensinogen gene is expressed and differentially regulated in multiple tissues of the rat. J Clin Invest. 1986 Jul;78(1):31-9.

Campbell SJ, Carlotti F, Hall PA, Clark AJ, Wolf CR. Regulation of the CYP1A1 promoter in transgenic mice: an exquisitely sensitive on-off system for cell specific gene regulation. J Cell Sci. 1996 Nov;109 (Pt 11):2619-25.

Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC. Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. Nature. 1994 Feb;367(6462):463-7.

Capponi AM. Distribution and signal transduction of angiotensin II AT1 and AT2 receptors. Blood Press Suppl. 1996;2:41-6.

Carmines PK, Navar LG. Disparate effects of Ca channel blockade on afferent and efferent arteriolar responses to ANG II. Am J Physiol. 1989 Jun;256(6 Pt 2):F1015-20.

Carmines PK, Perry MD, Hazelrig JB, Navar LG. Effects of preglomerular and postglomerular vascular resistance alterations on filtration fraction. Kidney Int Suppl. 1987 May;20:S229-32.

Carretero OA, Oparil S. Essential hypertension. Part I: definition and etiology. Circulation. 2000 Jan;101(3):329-35.

Castrop H, Höcherl K, Kurtz A, Schweda F, Todorov V, Wagner C. Physiology of kidney renin. Physiol Rev. 2010 Apr;90(2):607-73.

Chang SS, Grunder S, Hanukoglu A, Rösler A, Mathew PM, Hanukoglu I, Schild L, Lu Y, Shimkets RA, Nelson-Williams C, Rossier BC, Lifton RP. Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis, pseudohypoaldosteronism type 1. Nat Genet. 1996 Mar;12(3):248-53.

Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, Pearce D. Epithelial sodium channel regulated by aldosterone-induced protein sgk. Proc Natl Acad Sci U S A. 1999 Mar;96(5):2514-9.

Cheng ZJ, Finckenberg P, Louhelainen M, Merasto S, Tikkanen I, Vapaatalo H, Mervaala EM. Cardiovascular and renal effects of cyclooxygenase inhibition in transgenic rats harboring mouse renin-2 gene (TGR[mREN2]27). Eur J Pharmacol. 2003 Feb;461(2-3):159-69.

Cherradi N, Rossier MF, Vallotton MB, Timberg R, Friedberg I, Orly J, Wang XJ, Stocco DM, Capponi AM. Submitochondrial distribution of three key steroidogenic proteins (steroidogenic acute regulatory protein and cytochrome p450scc and 3beta-hydroxysteroid dehydrogenase isomerase enzymes) upon stimulation by intracellular calcium in adrenal glomerulosa cells. J Biol Chem. 1997 Mar;272(12):7899-907.

Chobanian AV. Shattuck Lecture. The hypertension paradox--more uncontrolled disease despite improved therapy. N Engl J Med. 2009 Aug 27;361(9):878-87. Erratum in: N Engl J Med. 2009 Oct;361(15):1516.

Clark AF, Sharp MG, Morley SD, Fleming S, Peters J, Mullins JJ. Renin-1 is essential for normal renal juxtaglomerular cell granulation and macula densa morphology. J Biol Chem. 1997 Jul;272(29):18185-90.

Coleman TG, Guyton AC. Hypertension caused by salt loading in the dog. 3. Onset transients of cardiac output and other circulatory variables. Circ Res. 1969 Aug;25(2):153-60.

Collins R, Peto R, MacMahon S, Hebert P, Fiebach NH, Eberlein KA, Godwin J, Qizilbash N, Taylor JO, Hennekens CH. Blood pressure, stroke, and coronary heart disease. Part 2, Short-term reductions in blood pressure: overview of randomised drug trials in their epidemiological context. Lancet. 1990 Apr;335(8693):827-38.

Connell JM, MacKenzie SM, Freel EM, Fraser R, Davies E. A lifetime of aldosterone excess: long-term consequences of altered regulation of aldosterone production for cardiovascular function. Endocr Rev. 2008 Apr;29(2):133-54.

Costa M, Majewski H. Facilitation of noradrenaline release from sympathetic nerves through activation of ACTH receptors, beta-adrenoceptors and angiotensin II receptors. Br J Pharmacol. 1988 Nov;95(3):993-1001.

Cowley AW Jr. Long-term control of arterial blood pressure. Physiol Rev. 1992 Jan;72(1):231-300.

Cox R, Bouzekri N, Martin S, Southam L, Hugill A, Golamaully M, Cooper R, Adeyemo A, Soubrier F, Ward R, Lathrop GM, Matsuda F, Farrall M. Angiotensin-1-converting enzyme (ACE) plasma concentration is influenced by multiple ACE-linked quantitative trait nucleotides. Hum Mol Genet. 2002 Nov;11(23):2969-77.

Crowley SD, Gurley SB, Herrera MJ, Ruiz P, Griffiths R, Kumar AP, Kim HS, Smithies O, Le TH, Coffman TM. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. Proc Natl Acad Sci USA. 2006 Nov;103(47):17985-90.

Cruz DN, Simon DB, Nelson-Williams C, Farhi A, Finberg K, Burleson L, Gill JR, Lifton RP. Mutations in the Na-Cl cotransporter reduce blood pressure in humans. Hypertension. 2001 Jun;37(6):1458-64.

Csikós T, Chung O, Unger T. Receptors and their classification: focus on angiotensin II and the AT2 receptor. J Hum Hypertens. 1998 May;12(5):311-8.

Curtis JJ, Luke RG, Dustan HP, Kashgarian M, Whelchel JD, Jones P, Diethelm AG. Remission of essential hypertension after renal transplantation. N Engl J Med. 1983 Oct;309(17):1009-15.

Davisson RL, Oliverio MI, Coffman TM, Sigmund CD. Divergent functions of angiotensin II receptor isoforms in the brain. J Clin Invest. 2000 Jul;106(1):103-6.

de Wardener HE. The primary role of the kidney and salt intake in the aetiology of essential hypertension: Part II. Clin Sci (Lond). 1990 Oct;79(4):289-97.

Debonneville C, Flores SY, Kamynina E, Plant PJ, Tauxe C, Thomas MA, Münster C, Chraïbi A, Pratt JH, Horisberger JD, Pearce D, Loffing J, Staub O. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na(+) channel cell surface expression. EMBO J. 2001 Dec;20(24):7052-9.

Dehmel B, Mervaala E, Lippoldt A, Gross V, Bohlender J, Ganten D, Luft FC. Pressure-natriuresis and diuresis in transgenic rats harboring both human renin and human angiotensinogen genes. J Am Soc Nephrol. 1998 Dec;9(12):2212-22.

Dickinson DP, Gross KW, Piccini N, Wilson CM. Evolution and variation of renin genes in mice. Genetics. 1984 Nov;108(3):651-67.

Ding Y, Davisson RL, Hardy DO, Zhu LJ, Merrill DC, Catterall JF, Sigmund CD. The kidney androgen-regulated protein promoter confers renal proximal tubule cell-specific and highly androgen-responsive expression on the human angiotensinogen gene in transgenic mice. J Biol Chem. 1997 Oct;272(44):28142-8.

Dixit MP, Xu L, Xu H, Bai L, Collins JF, Ghishan FK. Effect of angiotensin-II on renal Na+/H+ exchanger-NHE3 and NHE2. Biochim Biophys Acta. 2004 Jul;1664(1):38-44.

Dohi Y, Hahn AW, Boulanger CM, Bühler FR, Lüscher TF. Endothelin stimulated by angiotensin II augments contractility of spontaneously hypertensive rat resistance arteries. Hypertension. 1992 Feb;19(2):131-7.

Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE, Acton S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res. 2000 Sep;87(5):E1-9.

Dzau VJ, Bernstein K, Celermajer D, et al. Working Group on Tissue Angiotensin-converting enzyme, International Society of Cardiovascular Pharmacotherapy. The relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and endpoint data. Am J Cardiol. 2001 Nov;88(9A):1L-20L.

Dzau VJ, Burt DW, Pratt RE. Molecular biology of the renin-angiotensin system. Am J Physiol. 1988 Oct;255(4 Pt 2):F563-73.

Dzau VJ, Ellison KE, Brody T, Ingelfinger J, Pratt RE. A comparative study of the distributions of renin and angiotensinogen messenger ribonucleic acids in rat and mouse tissues. Endocrinology. 1987 Jun;120(6):2334-8.

Eaton DC, Malik B, Bao HF, Yu L, Jain L. Regulation of epithelial sodium channel trafficking by ubiquitination. Proc Am Thorac Soc. 2010 Feb;7(1):54-64.

Elfellah MS, Ogilvie RI. Effect of vasodilator drugs on coronary occlusion and reperfusion arrhythmias in anesthetized dogs. J Cardiovasc Pharmacol. 1985 Sep;7(5):826-32.

el-Sayed H, Goodall SR, Hainsworth R. Re-evaluation of Evans blue dye dilution method of plasma volume measurement. Clin Lab Haematol. 1995 Jun;17(2):189-94.

Engler S, Paul M, Pinto YM. The TGR(mRen2)27 transgenic rat model of hypertension. Regul Pept. 1998 Oct;77(1-3):3-8.

Epstein M. Aldosterone and the hypertensive kidney: its emerging role as a mediator of progressive renal dysfunction: a paradigm shift. J Hypertens. 2001 May;19(5):829-42.

Erbanová M, Thumová M, Husková Z, Vanecková I, Vanourková Z, Mullins JJ, Kramer HJ, Bürgelová M, Rakusan D, Cervenka L. Impairment of the autoregulation of renal hemodynamics and of the pressure-natriuresis relationship precedes the development of hypertension in Cyp1a1-Ren-2 transgenic rats. J Hypertens. 2009 Mar;27(3):575-86.

Erdös EG. Angiotensin I converting enzyme. Circ Res. 1975 Feb;36(2):247-55.

Faletti CJ, Perrotti N, Taylor SI, Blazer-Yost BL. sgk: an essential convergence point for peptide and steroid hormone regulation of ENaC-mediated Na+ transport. Am J Physiol Cell Physiol. 2002 Mar;282(3):C494-500.

Ferrario CM, Chappell MC, Tallant EA, Brosnihan KB, Diz DI. Counterregulatory actions of angiotensin-(1-7). Hypertension. 1997 Sep;30(3 Pt 2):535-41.

Field LJ, Gross KW. Ren-1 and Ren-2 loci are expressed in mouse kidney. Proc Natl Acad Sci U S A. 1985 Sep;82(18):6196-200.
Fink GD. Long-term sympatho-excitatory effect of angiotensin II: a mechanism of spontaneous and renovascular hypertension. Clin Exp Pharmacol Physiol. 1997 Jan;24(1):91-5.

Fitzsimons JT. Angiotensin, thirst, and sodium appetite. Physiol Rev. 1998 Jul;78(3):583-686.

Flatman PW. Cotransporters, WNKs and hypertension: important leads from the study of monogenetic disorders of blood pressure regulation. Clin Sci (Lond). 2007 Feb;112(4):203-16.

Fornage M, Amos CI, Kardia S, Sing CF, Turner ST, Boerwinkle E. Variation in the region of the angiotensin-converting enzyme gene influences interindividual

differences in blood pressure levels in young white males. Circulation. 1998 May ;97(18):1773-9.

Forrester LM, Henderson CJ, Glancey MJ, Back DJ, Park BK, Ball SE, Kitteringham NR, McLaren AW, Miles JS, Skett P, et al. Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. Biochem J. 1992 Jan ;281 (Pt 2):359-68.

Gaeggeler HP, Gonzalez-Rodriguez E, Jaeger NF, Loffing-Cueni D, Norregaard R, Loffing J, Horisberger JD, Rossier BC. Mineralocorticoid versus glucocorticoid receptor occupancy mediating aldosterone-stimulated sodium transport in a novel renal cell line. J Am Soc Nephrol. 2005 Apr;16(4):878-91.

Gainer JV, Hunley TE, Kon V, Nadeau JH, Muldowney JA 3rd, Brown NJ. Angiotensin II type I receptor polymorphism in African Americans lower frequency of the C1166 variant. Biochem Mol Biol Int. 1997 Sep;43(1):227-31.

Gamba G. Role of WNK kinases in regulating tubular salt and potassium transport and in the development of hypertension. Am J Physiol Renal Physiol. 2005 Feb;288(2):F245-52.

Gamba G, Saltzberg SN, Lombardi M, Miyanoshita A, Lytton J, Hediger MA, Brenner BM, Hebert SC. Primary structure and functional expression of a cDNA encoding the thiazide-sensitive, electroneutral sodium-chloride cotransporter. Proc Natl Acad Sci U S A. 1993 Apr;90(7):2749-53.

Garty H, Palmer LG. Epithelial sodium channels: function, structure, and regulation. Physiol Rev. 1997 Apr;77(2):359-96.

Garvin JL. Angiotensin stimulates bicarbonate transport and Na+/K+ ATPase in rat proximal straight tubules. J Am Soc Nephrol. 1991 Apr;1(10):1146-52.

Goldblatt H, Lynch J, Hanzal RF, Summerville WW. Studies on experimental hypertension : i. the production of persistent elevation of systolic blood pressure by means of renal ischemia. J Exp Med. 1934 Feb;59(3):347-79.

Goodfriend TL, Peach MJ. Angiotensin III: (DES-Aspartic Acid-1)-Angiotensin II. Evidence and speculation for its role as an important agonist in the renin - angiotensin system. Circ Res. 1975 Jun;36(6 Suppl 1):38-48.

Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A. 1992 Jun;89(12):5547-51.

Graciano ML, Mouton CR, Patterson ME, Seth DM, Mullins JJ, Mitchell KD. Renal vascular and tubulointerstitial inflammation and proliferation in Cyp1a1-Ren2 transgenic rats with inducible ANG II-dependent malignant hypertension. Am J Physiol Renal Physiol. 2007 Jun;292(6):F1858-66.

Greger R. Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. Physiol Rev. 1985 Jul;65(3):760-97.

Greger R. Physiology of renal sodium transport. Am J Med Sci. 2000 Jan;319(1):51-62.

Griendling KK, Lassègue B, Alexander RW. Angiotensin receptors and their therapeutic implications. Annu Rev Pharmacol Toxicol. 1996;36:281-306.

Griendling KK, Murphy TJ, Alexander RW. Molecular biology of the renin-angiotensin system. Circulation. 1993 Jun;87(6):1816-28.

Gross V, Lippoldt A, Schneider W, Luft FC. Effect of captopril and angiotensin II receptor blockade on pressure natriuresis in transgenic TGR(mRen-2)27 rats. Hypertension. 1995 Sep;26(3):471-9.

Gross V, Lippoldt A, Yagil C, Yagil Y, Luft FC. Pressure natriuresis in salt-sensitive and salt-resistant Sabra rats. Hypertension. 1997 Jun;29(6):1252-9.

Gross V, Luft FC. Exercising restraint in measuring blood pressure in conscious mice. Hypertension. 2003 Apr;41(4):879-81.

Gross V, Roman RJ, Cowley AW Jr. Abnormal pressure-natriuresis in transgenic renin gene rats. J Hypertens. 1994 Sep;12(9):1029-34.

Guan S, Fox J, Mitchell KD, Navar LG. Angiotensin and angiotensin converting enzyme tissue levels in two-kidney, one clip hypertensive rats. Hypertension. 1992 Dec;20(6):763-7.

Guidi E, Menghetti D, Milani S, Montagnino G, Palazzi P, Bianchi G. Hypertension may be transplanted with the kidney in humans: a long-term historical prospective follow-up of recipients grafted with kidneys coming from donors with or without hypertension in their families. J Am Soc Nephrol. 1996 Aug;7(8):1131-8.

Guyton AC. Blood pressure control--special role of the kidneys and body fluids. Science. 1991 Jun;252(5014):1813-6.

Guyton AC, Coleman TG, Cowley AV Jr, Scheel KW, Manning RD Jr, Norman RA Jr. Arterial pressure regulation. Overriding dominance of the kidneys in long-term regulation and in hypertension. Am J Med. 1972 May;52(5):584-94.

Hackenthal E, Paul M, Ganten D, Taugner R. Morphology, physiology, and molecular biology of renin secretion. Physiol Rev. 1990 Oct;70(4):1067-116.

Hall JE, Brands MW, Henegar JR. Angiotensin II and long-term arterial pressure regulation: the overriding dominance of the kidney. J Am Soc Nephrol. 1999 Apr;10 Suppl 12:S258-65.

Hall JE, Guyton AC, Mizelle HL. Role of the renin-angiotensin system in control of sodium excretion and arterial pressure. Acta Physiol Scand Suppl. 1990;591:48-62.

Haller H, Park JK, Dragun D, Lippoldt A, Luft FC. Leukocyte infiltration and ICAM-1 expression in two-kidney one-clip hypertension. Nephrol Dial Transplant. 1997 May;12(5):899-903.

Hamlyn JM, Blaustein MP. Sodium chloride, extracellular fluid volume, and blood pressure regulation. Am J Physiol. 1986 Oct;251(4 Pt 2):F563-75.

Handa RK, Ferrario CM, Strandhoy JW. Renal actions of angiotensin-(1-7): in vivo and in vitro studies. Am J Physiol. 1996 Jan;270(1 Pt 2):F141-7.

Harding JW, Felix D. The effects of the aminopeptidase inhibitors amastatin and bestatin on angiotensin-evoked neuronal activity in rat brain. Brain Res. 1987 Oct;424(2):299-304.

Harris PJ, Young JA. Dose-dependent stimulation and inhibition of proximal tubular sodium reabsorption by angiotensin II in the rat kidney. Pflugers Arch. 1977 Jan 17;367(3):295-7.

Hashimoto S, Huang Y, Mizel D, Briggs J, Schnermann J. Compensation of proximal tubule malabsorption in AQP1-deficient mice without TGF-mediated reduction of GFR. Acta Physiol Scand. 2004 Aug;181(4):455-62.

Hastie CE, Padmanabhan S, Dominiczak AF. Genome-Wide Association Studies of Hypertension: Light at the End of the Tunnel. International Journal of Hypertension. 2010 Apr;2010:509581

Hata A, Namikawa C, Sasaki M, Sato K, Nakamura T, Tamura K, Lalouel JM. Angiotensinogen as a risk factor for essential hypertension in Japan. J Clin Invest. 1994 Mar;93(3):1285-7.

Havlik RJ, Garrison RJ, Feinleib M, Kannel WB, Castelli WP, McNamara PM. Blood pressure aggregation in families. Am J Epidemiol. 1979 Sep;110(3):304-12.

Hebert SC, Desir G, Giebisch G, Wang W. Molecular diversity and regulation of renal potassium channels. Physiol Rev. 2005 Jan;85(1):319-71.

Hebert SC. Bartter syndrome. Curr Opin Nephrol Hypertens. 2003 Sep;12(5):527-32.

Heller J, Schubert G, Havlíckova J, Thurau K. The role of the kidney in the development of hypertension: a transplantation study in the Prague hypertensive rat. Pflugers Arch. 1993 Nov;425(3-4):208-12.

Herrmann HC, Dzau VJ. The feedback regulation of angiotensinogen production by components of the renin-angiotensin system. Circ Res. 1983 Mar;52(3):328-34.

Hodge G, Ye VZ, Duggan KA. Dysregulation of angiotensin II synthesis is associated with salt sensitivity in the spontaneous hypertensive rat. Acta Physiol Scand. 2002 Mar;174(3):209-15.

Horiuchi M. Functional aspects of angiotensin type 2 receptor. Adv Exp Med Biol. 1996;396:217-24.

Howard LL, Patterson ME, Mullins JJ, Mitchell KD. Salt-sensitive hypertension develops after transient induction of ANG II-dependent hypertension in Cyp1a1-Ren2 transgenic rats. Am J Physiol Renal Physiol. 2005 Apr;288(4):F810-5.

Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, Boucher R, Rossier BC. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. Nat Genet. 1996 Mar;12(3):325-8.

Hunyady L, Catt KJ. Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. Mol Endocrinol. 2006 May;20(5):953-70.

Husková Z, Kramer H, Vanourková Z, Thumová M, Malý J, Opocenský M, Skaroupková P, Kolský A, Vernerová Z, Cervenka L. Effects of dietary salt load and salt depletion on the course of hypertension and angiotensin II levels in male and female heterozygous Ren-2 transgenic rats. Kidney Blood Press Res. 2007;30(1):45-55.

Husková Z, Vanourková Z, Erbanová M, Thumová M, Opocenský M, Mullins JJ, Kramer HJ, Bürgelová M, Cervenka L. Inappropriately high circulating and intrarenal angiotensin II levels during dietary salt loading exacerbate hypertension in Cyp1a1-Ren-2 transgenic rats. J Hypertens. 2010 Mar;28(3):495-509.

Ibrahim MM. RAS inhibition in hypertension. J Hum Hypertens. 2006 Feb;20(2):101-8.

Ichikawa I, Brenner BM. Importance of efferent arteriolar vascular tone in regulation of proximal tubule fluid reabsorption and glomerulotubular balance in the rat. J Clin Invest. 1980 May;65(5):1192-201.

Imamura A, Mackenzie HS, Lacy ER, Hutchison FN, Fitzgibbon WR, Ploth DW. Effects of chronic treatment with angiotensin converting enzyme inhibitor or an angiotensin receptor antagonist in two-kidney, one-clip hypertensive rats. Kidney Int. 1995 May;47(5):1394-402.

Irvine RJ, White J, Chan R. The influence of restraint on blood pressure in the rat. J Pharmacol Toxicol Methods. 1997 Nov;38(3):157-62.

Ito M, Oliverio MI, Mannon PJ, Best CF, Maeda N, Smithies O, Coffman TM. Regulation of blood pressure by the type 1A angiotensin II receptor gene. Proc Natl Acad Sci U S A. 1995 Apr;92(8):3521-5.

Ito S, Arima S, Ren YL, Juncos LA, Carretero OA. Endothelium-derived relaxing factor/nitric oxide modulates angiotensin II action in the isolated microperfused rabbit afferent but not efferent arteriole. J Clin Invest. 1993 May;91(5):2012-9.

Jaramillo HN, Sambhi MP, Bouhnik J, Corvol P, Menard J. Liver angiotensinogen synthesis and release during captopril treatment in sodium-depleted rats. Endocrinology. 1987 Apr;120(4):1384-90.

Jellinck PH, Forkert PG, Riddick DS, Okey AB, Michnovicz JJ, Bradlow HL. Ah receptor binding properties of indole carbinols and induction of hepatic estradiol hydroxylation. Biochem Pharmacol. 1993 Mar;45(5):1129-36.

Jeunemaitre X, Soubrier F, Kotelevtsev YV, Lifton RP, Williams CS, Charru A, Hunt SC, Hopkins PN, Williams RR, Lalouel JM, et al. Molecular basis of human hypertension: role of angiotensinogen. Cell. 1992 Oct;71(1):169-80.

Johns DW, Baker KM, Ayers CR, Vaughan ED Jr, Carey RM, Peach MJ, Yancey MR, Ortt EM, Williams SC. Acute and chronic effect of captropril in hypertensive patients. Hypertension. 1980 Jul-Aug;2(4):567-75.

Julius S, Nesbitt S. Sympathetic overactivity in hypertension. A moving target. Am J Hypertens. 1996 Nov;9(11):113S-120S.

Kahle KT, Wilson FH, Leng Q, Lalioti MD, O'Connell AD, Dong K, Rapson AK, MacGregor GG, Giebisch G, Hebert SC, Lifton RP. WNK4 regulates the balance between renal NaCl reabsorption and K+ secretion. Nat Genet. 2003 Dec;35(4):372-6.

Kainulainen K, Perola M, Terwilliger J, Kaprio J, Koskenvuo M, Syvänen AC, Vartiainen E, Peltonen L, Kontula K. Evidence for involvement of the type 1 angiotensin II receptor locus in essential hypertension. Hypertension. 1999 Mar;33(3):844-9.

Kamynina E, Debonneville C, Hirt RP, Staub O. Liddle's syndrome: a novel mouse Nedd4 isoform regulates the activity of the epithelial Na(+) channel. Kidney Int. 2001 Aug;60(2):466-71.

Kanagy NL, Pawloski CM, Fink GD. Role of aldosterone in angiotensin II-induced hypertension in rats. Am J Physiol. 1990 Jul;259(1 Pt 2):R102-9.

Kantachuvesiri S, Fleming S, Peters J, Peters B, Brooker G, Lammie AG, McGrath I, Kotelevtsev Y, Mullins JJ. Controlled hypertension, a transgenic toggle switch reveals differential mechanisms underlying vascular disease. J Biol Chem. 2001 Sep;276(39):36727-33.

Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. Lancet. 2005 Jan;365(9455):217-23.

Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA. The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. Proc Natl Acad Sci U S A. 1998 Nov;95(24):14552-7.

Kline RL, Liu F. Modification of pressure natriuresis by long-term losartan in spontaneously hypertensive rats. Hypertension. 1994 Oct;24(4):467-73.

Kobayashi M, Furukawa Y, Chiba S. Positive chronotropic and inotropic effects of angiotensin II in the dog heart. Eur J Pharmacol. 1978 Jul;50(1):17-25.

Kobayashi T, Cohen P. Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. Biochem J. 1999 Apr;339 (Pt 2):319-28.

Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. Pharmacol Rev. 2007 Sep;59(3):251-87.

Kojima I, Ogata E. Direct demonstration of adrenocorticotropin-induced changes in cytoplasmic free calcium with aequorin in adrenal glomerulosa cell. J Biol Chem. 1986 Jul;261(21):9832-8.

Komlosi P, Fuson AL, Fintha A, Peti-Peterdi J, Rosivall L, Warnock DG, Bell PD. Angiotensin I conversion to angiotensin II stimulates cortical collecting duct sodium transport. Hypertension. 2003 Aug;42(2):195-9.

Komlosi P, Fintha A, Bell PD. Current mechanisms of macula densa cell signalling. Acta Physiol Scand. 2004 Aug;181(4):463-9.

Kramer K, Kinter L, Brockway BP, Voss HP, Remie R, Van Zutphen BL. The use of radiotelemetry in small laboratory animals: recent advances. Contemp Top Lab Anim Sci. 2001 Jan;40(1):8-16.

Kramer K, Kinter LB. Evaluation and applications of radiotelemetry in small laboratory animals. Physiol Genomics. 2003 May;13(3):197-205.

Krege JH, Hodgin JB, Hagaman JR, Smithies O. A noninvasive computerized tail-cuff system for measuring blood pressure in mice. Hypertension. 1995 May;25(5):1111-5.

Kurtz TW, Griffin KA, Bidani AK, Davisson RL, Hall JE. Recommendations for blood pressure measurement in humans and experimental animals: part 2: blood pressure measurement in experimental animals: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart

Association Council on High Blood Pressure Research. Arterioscler Thromb Vasc Biol. 2005 Mar;25(3):e22-33.

Kwon TH, Nielsen J, Kim YH, Knepper MA, Frøkiaer J, Nielsen S. Regulation of sodium transporters in the thick ascending limb of rat kidney: response to angiotensin II. Am J Physiol Renal Physiol. 2003 Jul;285(1):F152-65.

Lalioti MD, Zhang J, Volkman HM, Kahle KT, Hoffmann KE, Toka HR, Nelson-Williams C, Ellison DH, Flavell R, Booth CJ, Lu Y, Geller DS, Lifton RP. Wnk4 controls blood pressure and potassium homeostasis via regulation of mass and activity of the distal convoluted tubule. Nat Genet. 2006 Oct;38(10):1124-32.

Lawes CM, Vander Hoorn S, Rodgers A; International Society of Hypertension. Global burden of blood-pressure-related disease, 2001. Lancet. 2008 May;371(9623):1513-8.

Lee DH, Riquier AD, Yang LE, Leong PK, Maunsbach AB, McDonough AA. Acute hypertension provokes acute trafficking of distal tubule Na-Cl cotransporter (NCC) to subapical cytoplasmic vesicles. Am J Physiol Renal Physiol. 2009 Apr;296(4):F810-8.

Lee MA, Böhm M, Paul M, Bader M, Ganten U, Ganten D. Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. Am J Physiol. 1996 Jun;270(6 Pt 1):E919-29.

Lemarié CA, Paradis P, Schiffrin EL. New insights on signaling cascades induced by cross-talk between angiotensin II and aldosterone. J Mol Med. 2008 Jun;86(6):673-8.

Lemos VS, Silva DM, Walther T, Alenina N, Bader M, Santos RA. The endothelium-dependent vasodilator effect of the nonpeptide Ang(1-7) mimic AVE 0991 is abolished in the aorta of mas-knockout mice. J Cardiovasc Pharmacol. 2005 Sep;46(3):274-9.

Levy D, DeStefano AL, Larson MG, O'Donnell CJ, Lifton RP, Gavras H, Cupples LA, Myers RH. Evidence for a gene influencing blood pressure on chromosome 17. Genome scan linkage results for longitudinal blood pressure phenotypes in subjects from the framingham heart study. Hypertension. 2000 Oct;36(4):477-83.

Levy D, Ehret GB, Rice K, et al Genome-wide association study of blood pressure and hypertension. Nat Genet. 2009 Jun;41(6):677-87.

Li J, Brasier AR. Angiotensinogen gene activation by angiotensin II is mediated by the rel A (nuclear factor-kappaB p65) transcription factor: one mechanism for the renin angiotensin system positive feedback loop in hepatocytes. Mol Endocrinol. 1996 Mar;10(3):252-64.

Li Q, Zhang J, Pfaffendorf M, van Zwieten PA. Direct positive chronotropic effects of angiotensin II and angiotensin III in pithed rats and in rat isolated atria. Br J Pharmacol. 1996 Aug;118(7):1653-8.

Liddle GW, Bledsoe T, Coppage WS. A familial renal disorder simulating primary aldosteronism but with negligible aldosterone secretion. Trans Assoc Am Physicians. 1963;76:199-213.

Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. Cell. 2001 Feb;104(4):545-56.

Lifton RP. Molecular genetics of human blood pressure variation. Science. 1996 May;272(5262):676-80.

Liu X, Bellamy CO, Bailey MA, Mullins LJ, Dunbar DR, Kenyon CJ, Brooker G, Kantachuvesiri S, Maratou K, Ashek A, Clark AF, Fleming S, Mullins JJ. Angiotensin-converting enzyme is a modifier of hypertensive end organ damage. J Biol Chem. 2009 Jun;284(23):15564-72.

Lombardi D, Gordon KL, Polinsky P, Suga S, Schwartz SM, Johnson RJ. Salt-sensitive hypertension develops after short-term exposure to Angiotensin II. Hypertension. 1999 Apr;33(4):1013-9.

Longini IM Jr, Higgins MW, Hinton PC, Moll PP, Keller JB. Environmental and genetic sources of familial aggregation of blood pressure in Tecumseh, Michigan. Am J Epidemiol. 1984 Jul;120(1):131-44.

Lorenz JN, Schultheis PJ, Traynor T, Shull GE, Schnermann J. Micropuncture analysis of single-nephron function in NHE3-deficient mice. Am J Physiol. 1999 Sep;277(3 Pt 2):F447-53.

Lorenz JN. A practical guide to evaluating cardiovascular, renal, and pulmonary function in mice. Am J Physiol Regul Integr Comp Physiol. 2002 Jun;282(6):R1565-82.

Loub WD, Wattenberg LW, Davis DW. Aryl hydrocarbon hydroxylase induction in rat tissues by naturally occurring indoles of cruciferous plants. J Natl Cancer Inst. 1975 Apr;54(4):985-8.

Lu M, Wang T, Yan Q, Yang X, Dong K, Knepper MA, Wang W, Giebisch G, Shull GE, Hebert SC. Absence of small conductance K+ channel (SK) activity in apical membranes of thick ascending limb and cortical collecting duct in ROMK (Bartter's) knockout mice. J Biol Chem. 2002 Oct;277(40):37881-7.

Luft FC. Twins in cardiovascular genetic research. Hypertension. 2001 Feb;37(2 Part 2):350-6.

Ma J, Weisberg A, Griffin JP, Vaughan DE, Fogo AB, Brown NJ. Plasminogen activator inhibitor-1 deficiency protects against aldosterone-induced glomerular injury. Kidney Int. 2006 Mar;69(6):1064-72.

MacDonald P, MacKenzie S, Ramage LE, Seckl JR, Brown RW. Corticosteroid regulation of amiloride-sensitive sodium-channel subunit mRNA expression in mouse kidney. J Endocrinol. 2000 Apr;165(1):25-37.

Machnik A, Neuhofer W, Jantsch J, Dahlmann A, Tammela T, Machura K, Park JK, Beck FX, Müller DN, Derer W, Goss J, Ziomber A, Dietsch P, Wagner H, van Rooijen N, Kurtz A, Hilgers KF, Alitalo K, Eckardt KU, Luft FC, Kerjaschki D, Titze J. Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. Nat Med. 2009 May;15(5):545-52.

Manunta P, Burnier M, D'Amico M, Buzzi L, Maillard M, Barlassina C, Lanella G, Cusi D, Bianchi G. Adducin polymorphism affects renal proximal tubule reabsorption in hypertension. Hypertension. 1999 Feb;33(2):694-7.

Martinez DV, Rocha R, Matsumura M, Oestreicher E, Ochoa-Maya M, Roubsanthisuk W, Williams GH, Adler GK. Cardiac damage prevention by eplerenone: comparison with low sodium diet or potassium loading. Hypertension. 2002 Feb;39(2 Pt 2):614-8.

Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA. Aldosterone-mediated regulation of ENaC alpha, beta, and gamma subunit proteins in rat kidney. J Clin Invest. 1999 Oct;104(7):R19-23.

Masilamani S, Wang X, Kim GH, Brooks H, Nielsen J, Nielsen S, Nakamura K, Stokes JB, Knepper MA. Time course of renal Na-K-ATPase, NHE3, NKCC2, NCC, and ENaC abundance changes with dietary NaCl restriction. Am J Physiol Renal Physiol. 2002 Oct;283(4):F648-57.

Mattson DL, Raff H, Roman RJ. Influence of angiotensin II on pressure natriuresis and renal hemodynamics in volume-expanded rats. Am J Physiol. 1991 Jun;260(6 Pt 2):R1200-9.

May A, Puoti A, Gaeggeler HP, Horisberger JD, Rossier BC. Early effect of aldosterone on the rate of synthesis of the epithelial sodium channel alpha subunit in A6 renal cells. J Am Soc Nephrol. 1997 Dec;8(12):1813-22.

McDonald FJ, Yang B, Hrstka RF, Drummond HA, Tarr DE, McCray PB Jr, Stokes JB, Welsh MJ, Williamson RA. Disruption of the beta subunit of the epithelial Na+ channel in mice: hyperkalemia and neonatal death associated with a

pseudohypoaldosteronism phenotype. Proc Natl Acad Sci U S A. 1999 Feb;96(4):1727-31.

McDonough AA, Leong PK, Yang LE. Mechanisms of pressure natriuresis: how blood pressure regulates renal sodium transport. Ann N Y Acad Sci. 2003 Apr;986:669-77.

McKinley MJ, Albiston AL, Allen AM, Mathai ML, May CN, McAllen RM, Oldfield BJ, Mendelsohn FA, Chai SY. The brain renin-angiotensin system: location and physiological roles. Int J Biochem Cell Biol. 2003 Jun;35(6):901-18.

Melander O, Orho-Melander M, Bengtsson K, Lindblad U, Råstam L, Groop L, Hulthén UL. Genetic variants of thiazide-sensitive NaCl-cotransporter in Gitelman's syndrome and primary hypertension. Hypertension. 2000 Sep;36(3):389-94.

Meneton P, Ichikawa I, Inagami T, Schnermann J. Renal physiology of the mouse. Am J Physiol Renal Physiol. 2000 Mar;278(3):F339-51.

Mitchell KD, Bagatell SJ, Miller CS, Mouton CR, Seth DM, Mullins JJ. Genetic clamping of renin gene expression induces hypertension and elevation of intrarenal Ang II levels of graded severity in Cyp1a1-Ren2 transgenic rats. J Renin Angiotensin Aldosterone Syst. 2006 Jun;7(2):74-86.

Mitchell KD, Braam B, Navar LG. Hypertensinogenic mechanisms mediated by renal actions of renin-angiotensin system. Hypertension. 1992 Jan;19(1 Suppl):I18-27.

Mitchell KD, Jacinto SM, Mullins JJ. Proximal tubular fluid, kidney, and plasma levels of angiotensin II in hypertensive ren-2 transgenic rats. Am J Physiol. 1997 Aug;273(2 Pt 2):F246-53.

Mitchell KD, Mullins JJ. ANG II dependence of tubuloglomerular feedback responsiveness in hypertensive ren-2 transgenic rats. Am J Physiol. 1995 May;268(5 Pt 2):F821-8.

Mitchell KD, Mullins JJ. Enhanced tubuloglomerular feedback in Cyp1a1-Ren2 transgenic rats with inducible ANG II-dependent malignant hypertension. Am J Physiol Renal Physiol. 2005 Dec;289(6):F1210-6.

Mitchell KD, Navar LG. Enhanced tubuloglomerular feedback during peritubular infusions of angiotensins I and II. Am J Physiol. 1988 Sep;255(3 Pt 2):F383-90.

Mongeau JG, Biron P, Sing CF. The influence of genetics and household environment upon the variability of normal blood pressure: the Montreal Adoption Survey. Clin Exp Hypertens A. 1986;8(4-5):653-60.

Morimoto A, Uzu T, Fujii T, Nishimura M, Kuroda S, Nakamura S,Inenaga T, Kimura G. Sodium sensitivity and cardiovascular events in patients with essential hypertension. Lancet. 1997 Dec;350(9093):1734-7.

Morimoto S, Cassell MD, Beltz TG, Johnson AK, Davisson RL, Sigmund CD. Elevated blood pressure in transgenic mice with brain-specific expression of human angiotensinogen driven by the glial fibrillary acidic protein promoter. Circ Res. 2001 Aug;89(4):365-72.

Morimoto S, Sigmund CD. Angiotensin mutant mice: a focus on the brain renin-angiotensin system. Neuropeptides. 2002 Apr-Jun;36(2-3):194-200.
Morgan L, Broughton Pipkin F, Kalsheker N. Angiotensinogen: molecular biology, biochemistry and physiology. Int J Biochem Cell Biol. 1996 Nov;28(11):1211-22.

Muehrcke RC, Rosen S. Hypokalemic nephropathy in rat and man: a light and electron microscopic study. Lab Invest. 1964 Nov;13:1359-73.

Müller J. Aldosterone: the minority hormone of the adrenal cortex. Steroids. 1995 Jan;60(1):2-9.

Mullins JJ, Burt DW, Windass JD, McTurk P, George H, Brammar WJ. Molecular cloning of two distinct renin genes from the DBA/2 mouse. EMBO J. 1982;1(11):1461-6.

Mullins JJ, Peters J, Ganten D. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. Nature. 1990 Apr;344(6266):541-4.

Mullins LJ, Bailey MA, Mullins JJ. Hypertension, kidney, and transgenics: a fresh perspective. Physiol Rev. 2006 Apr;86(2):709-46.

Mullins LJ, Payne CM, Kotelevtseva N, Brooker G, Fleming S, Harris S, Mullins JJ. Granulation rescue and developmental marking of juxtaglomerular cells using "piggy-BAC" recombination of the mouse ren locus. J Biol Chem. 2000 Dec;275(51):40378-84.

Munday KA, Parsons BJ, Poat JA. The effect of angiotensin on cation transport by rat kidney cortex slices. J Physiol. 1971 May;215(1):269-82.

Naray-Fejes-Toth A, Canessa C, Cleaveland ES, Aldrich G, Fejes-Tóth G. sgk is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial na+ channels. J Biol Chem. 1999 Jun;274(24):16973-8.

Nasjletti A, Masson GM. Stimulation of angiotensinogen formation by renin and angiotensin. Proc Soc Exp Biol Med. 1973 Jan;142(1):307-10.

Navar LG. The kidney in blood pressure regulation and development of hypertension. Med Clin North Am. 1997 Sep;81(5):1165-98.

Navar LG, Imig JD, Zou L, Wang CT. Intrarenal production of angiotensin II. Semin Nephrol. 1997 Sep;17(5):412-22.

Navar LG, Inscho EW, Majid SA, Imig JD, Harrison-Bernard LM, Mitchell KD. Paracrine regulation of the renal microcirculation. Physiol Rev. 1996 Apr;76(2):425-536.

Navar LG, Majid DS. Interactions between arterial pressure and sodium excretion. Curr Opin Nephrol Hypertens. 1996 Jan;5(1):64-71.
Navar LG, Paul RV, Carmines PK, Chou CL, Marsh DJ. Intrarenal mechanisms mediating pressure natriuresis: role of angiotensin and prostaglandins. Fed Proc. 1986 Dec;45(13):2885-91.

Navar LG, Prieto-Carrasquero MC, Kobori H. Molecular aspects of the renal renin-angiotensin system. In: Re RN, Dipette DJ, Schilffrin EL, Sowers JR. Molecular Mechanisms in Hypertension. London: Taylor and Francis; 2006:3–14.

Navar LG, Zou L, Von Thun A, Tarng Wang C, Imig JD, Mitchell KD. Unraveling the Mystery of Goldblatt Hypertension. News Physiol Sci. 1998 Aug;13:170-176.

Newton-Cheh C, Johnson T, Gateva V, *et al.* Genome-wide association study identifies eight loci associated with blood pressure. Nat Genet. 2009 Jun;41(6):666-76.

Nielsen J, Kwon TH, Frøkiaer J, Knepper MA, Nielsen S. Maintained ENaC trafficking in aldosterone-infused rats during mineralocorticoid and glucocorticoid receptor blockade. Am J Physiol Renal Physiol. 2007 Jan;292(1):F382-94.

Nielsen J, Kwon TH, Masilamani S, Beutler K, Hager H, Nielsen S, Knepper MA. Sodium transporter abundance profiling in kidney: effect of spironolactone. Am J Physiol Renal Physiol. 2002 Nov;283(5):F923-33.

Nishiyama A, Yao L, Nagai Y, Miyata K, Yoshizumi M, Kagami S, Kondo S, Kiyomoto H, Shokoji T, Kimura S, Kohno M, Abe Y. Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. Hypertension. 2004 Apr;43(4):841-8.

O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ, Myers RH, Levy D. Evidence for association and genetic linkage of the angiotensin-converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study. Circulation. 1998 May;97(18):1766-72.

Onoyama K, Omae T, Inagami T. Tissue edema and arterial lesions produced by pure submaxillary gland renin of mouse. Jpn Heart J. 1978 Jul;19(4):522-30.

Opay AL, Mouton CR, Mullins JJ, Mitchell KD. Cyclooxygenase-2 inhibition normalizes arterial blood pressure in CYP1A1-REN2 transgenic rats with inducible

ANG II-dependent malignant hypertension. Am J Physiol Renal Physiol. 2006 Sep;291(3):F612-8.

Ortiz RM, Graciano ML, Mullins JJ, Mitchell KD. Aldosterone receptor antagonism alleviates proteinuria, but not malignant hypertension, in Cyp1a1-Ren2 transgenic rats. Am J Physiol Renal Physiol. 2007a Nov;293(5):F1584-91.

Ortiz RM, Graciano ML, Seth D, Awayda MS, Navar LG. Aldosterone receptor antagonism exacerbates intrarenal angiotensin II augmentation in ANG II-dependent hypertension. Am J Physiol Renal Physiol. 2007b Jul;293(1):F139-47.

Osborn JW, Ariza-Nieto P, Collister JP, Soucheray S, Zimmerman B, Katz S. Responsiveness vs. basal activity of plasma ANG II as a determinant of arterial pressure salt sensitivity. Am J Physiol Heart Circ Physiol. 2003 Nov;285(5):H2142-9.

Ozono R, Wang ZQ, Moore AF, Inagami T, Siragy HM, Carey RM. Expression of the subtype 2 angiotensin (AT2) receptor protein in rat kidney. Hypertension. 1997 Nov;30(5):1238-46.

Paton JF, Wang S, Polson JW, Kasparov S. Signalling across the blood brain barrier by angiotensin II: novel implications for neurogenic hypertension. J Mol Med. 2008 Jun;86(6):705-10.

Patterson ME, Mouton CR, Mullins JJ, Mitchell KD. Interactive effects of superoxide anion and nitric oxide on blood pressure and renal hemodynamics in transgenic rats with inducible malignant hypertension. Am J Physiol Renal Physiol. 2005 Oct;289(4):F754-9.

Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. Physiol Rev. 2006 Jul;86(3):747-803.

Peach MJ. Renin-angiotensin system: biochemistry and mechanisms of action. Physiol Rev. 1977 Apr;57(2):313-70.

Pearce D. SGK1 regulation of epithelial sodium transport. Cell Physiol Biochem. 2003;13(1):13-20.

Pelkonen O, Nebert DW. Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. Pharmacol Rev. 1982 Jun;34(2):189-222.

Pentz ES, Lopez ML, Kim HS, Carretero O, Smithies O, Gomez RA. Ren1d and Ren2 cooperate to preserve homeostasis: evidence from mice expressing GFP in place of Ren1d. Physiol Genomics. 2001 Jun;6(1):45-55.

Peters B, Clausmeyer S, Obermüller N, Woyth A, Kränzlin B, Gretz N, Peters J. Specific regulation of StAR expression in the rat adrenal zona glomerulosa. An in situ hybridization study. J Histochem Cytochem. 1998 Nov;46(11):1215-21.

Peti-Peterdi J, Warnock DG, Bell PD. Angiotensin II directly stimulates ENaC activity in the cortical collecting duct via AT(1) receptors. J Am Soc Nephrol. 2002 May;13(5):1131-5.

Phillips MI, Sumners C. Angiotensin II in central nervous system physiology. Regul Pept. 1998 Nov;78(1-3):1-11.

Pinto R, Gradman AH. Direct renin inhibition: an update. Curr Hypertens Rep. 2009 Dec;11(6):456-62.

Ploth DW. Angiotensin-dependent renal mechanisms in two-kidney, one-clip renal vascular hypertension. Am J Physiol. 1983 Aug;245(2):F131-41.
Pobiner BF, Hewlett EL, Garrison JC. Role of Ni in coupling angiotensin receptors to inhibition of adenylate cyclase in hepatocytes. J Biol Chem. 1985 Dec;260(30):16200-9.

Popovic V. Adaptation to restraint in the rat. Physiologist. 1988;31(1 Suppl):S65-6.

Price HL. General anesthesia and circulatory homeostasis. Physiol Rev. 1960 Apr;40:187-218.

Pu Q, Amiri F, Gannon P, Schiffrin EL. Dual angiotensin-converting enzyme/neutral endopeptidase inhibition on cardiac and renal fibrosis and inflammation in DOCA-salt hypertensive rats. J Hypertens. 2005 Feb;23(2):401-9.

Reid IA. Interactions between ANG II, sympathetic nervous system, and baroreceptor reflexes in regulation of blood pressure. Am J Physiol. 1992 Jun;262(6 Pt 1):E763-78.

Ren Y, Garvin JL, Carretero OA. Vasodilator action of angiotensin-(1-7) on isolated rabbit afferent arterioles. Hypertension. 2002 Mar;39(3):799-802.

Rettig R, Stauss H, Folberth C, Ganten D, Waldherr B, Unger T. Hypertension transmitted by kidneys from stroke-prone spontaneously hypertensive rats. Am J Physiol. 1989 Aug;257(2 Pt 2):F197-203.

Rice GI, Thomas DA, Grant PJ, Turner AJ, Hooper NM. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. Biochem J. 2004 Oct;383(Pt 1):45-51.

Richardson C, Rafiqi FH, Karlsson HK, Moleleki N, Vandewalle A, Campbell DG, Morrice NA, Alessi DR. Activation of the thiazide-sensitive Na+-Cl- cotransporter by the WNK-regulated kinases SPAK and OSR1. J Cell Sci. 2008 Mar;121(Pt 5):675-84.

Riemenschneider T, Bohle A. Morphologic aspects of low-potassium and low-sodium nephropathy. Clin Nephrol. 1983 Jun;19(6):271-9.

Rocha R, Martin-Berger CL, Yang P, Scherrer R, Delyani J, McMahon E. Selective aldosterone blockade prevents angiotensin II/salt-induced vascular inflammation in the rat heart. Endocrinology. 2002 Dec;143(12):4828-36.

Rocha R, Stier CT Jr, Kifor I, Ochoa-Maya MR, Rennke HG, Williams GH, Adler GK. Aldosterone: a mediator of myocardial necrosis and renal arteriopathy. Endocrinology. 2000 Oct;141(10):3871-8.

Rodríguez-Iturbe B, Quiroz Y, Ferrebuz A, Parra G, Vaziri ND. Evolution of renal interstitial inflammation and NF-kappaB activation in spontaneously hypertensive rats. Am J Nephrol. 2004 Nov-Dec;24(6):587-94.

Rodríguez-Iturbe B, Quiroz Y, Nava M, Bonet L, Chávez M, Herrera-Acosta J, Johnson RJ, Pons HA. Reduction of renal immune cell infiltration results in blood pressure control in genetically hypertensive rats. Am J Physiol Renal Physiol. 2002 Feb;282(2):F191-201.

Roman RJ. Abnormal renal hemodynamics and pressure-natriuresis relationship in Dahl salt-sensitive rats. Am J Physiol. 1986 Jul;251(1 Pt 2):F57-65.

Roman RJ, Cowley AW Jr, Garcia-Estañ J, Lombard JH. Pressure-diuresis in volume-expanded rats. Cortical and medullary hemodynamics. Hypertension. 1988 Aug;12(2):168-76.

Roman RJ, Cowley AW Jr. Abnormal pressure-diuresis-natriuresis response in spontaneously hypertensive rats. Am J Physiol. 1985 Feb;248(2 Pt 2):F199-205.

Roman RJ, Kaldunski M. Pressure natriuresis and cortical and papillary blood flow in inbred Dahl rats. Am J Physiol. 1991 Sep;261(3 Pt 2):R595-602.

Rossi GP, Sacchetto A, Cesari M, Pessina AC. Interactions between endothelin-1 and the renin-angiotensin-aldosterone system. Cardiovasc Res. 1999 Aug;43(2):300-7.

Rossier BC, Pradervand S, Schild L, Hummler E. Epithelial sodium channel and the control of sodium balance: interaction between genetic and environmental factors. Annu Rev Physiol. 2002;64:877-97.

San-Cristobal P, Pacheco-Alvarez D, Richardson C, Ring AM, Vazquez N, Rafiqi FH, Chari D, Kahle KT, Leng Q, Bobadilla NA, Hebert SC, Alessi DR, Lifton RP, Gamba G. Angiotensin II signaling increases activity of the renal Na-Cl cotransporter through a WNK4-SPAK-dependent pathway. Proc Natl Acad Sci. 2009 Mar;106(11):4384-9.

Sandberg MB, Maunsbach AB, McDonough AA. Redistribution of distal tubule Na+-Cl- cotransporter (NCC) in response to a high-salt diet. Am J Physiol Renal Physiol. 2006 Aug;291(2):F503-8.

Sandberg MB, Riquier AD, Pihakaski-Maunsbach K, McDonough AA, Maunsbach AB. ANG II provokes acute trafficking of distal tubule Na+-Cl(-) cotransporter to apical membrane. Am J Physiol Renal Physiol. 2007 Sep;293(3):F662-9.
Sander M, Bader M, Djavidani B, Maser-Gluth C, Vecsei P, Mullins J, Ganten D, Peters J. The role of the adrenal gland in hypertensive transgenic rat TGR(mREN2)27. Endocrinology. 1992 Aug;131(2):807-14.

Sato Y, Ogata E, Fujita T. Role of chloride in angiotensin II-induced salt-sensitive hypertension. Hypertension. 1991 Nov;18(5):622-9.

Schild L, Lu Y, Gautschi I, Schneeberger E, Lifton RP, Rossier BC. Identification of a PY motif in the epithelial Na channel subunits as a target sequence for mutations causing channel activation found in Liddle syndrome. EMBO J. 1996 May;15(10):2381-7.

Schlatter E, Bleich M, Hirsch J, Greger R. pH-sensitive K+ channels in the distal nephron. Nephrol Dial Transplant. 1993;8(6):488-90.

Schlatter E, Haxelmans S, Ankorina I, Kleta R. Regulation of Na+/H+ exchange by diadenosine polyphosphates, angiotensin II, and vasopressin in rat cortical collecting duct. J Am Soc Nephrol. 1995 Oct;6(4):1223-9.

Schnermann J. Sodium transport deficiency and sodium balance in gene-targeted mice. Acta Physiol Scand. 2001 Sep;173(1):59-66.

Schnermann J, Chou CL, Ma T, Traynor T, Knepper MA, Verkman AS. Defective proximal tubular fluid reabsorption in transgenic aquaporin-1 null mice. Proc Natl Acad Sci U S A. 1998a Aug 4;95(16):9660-4.

Schnermann J. Juxtaglomerular cell complex in the regulation of renal salt excretion. Am J Physiol. 1998b Feb;274(2 Pt 2):R263-79.

Schnermann JB, Traynor T, Yang T, Huang YG, Oliverio MI, Coffman T, Briggs JP. Absence of tubuloglomerular feedback responses in AT1A receptor-deficient mice. Am J Physiol. 1997 Aug;273(2 Pt 2):F315-20.

Schultheis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, Giebisch G, Aronson PS, Lorenz JN, Shull GE. Renal and intestinal absorptive defects in mice lacking the NHE3 Na+/H+ exchanger. Nat Genet. 1998a Jul;19(3):282-5.

Schultheis PJ, Lorenz JN, Meneton P, Nieman ML, Riddle TM, Flagella M, Duffy JJ, Doetschman T, Miller ML, Shull GE. Phenotype resembling Gitelman's syndrome in mice lacking the apical Na+-Cl- cotransporter of the distal convoluted tubule. J Biol Chem. 1998b Oct 30;273(44):29150-5.Sep;297(3):F704-12.
.

Sharma M, Sharma R, Greene AS, McCarthy ET, Savin VJ. Documentation of angiotensin II receptors in glomerular epithelial cells. Am J Physiol. 1998 Mar;274(3 Pt 2):F623-7.

Sharp MG, Fettes D, Brooker G, Clark AF, Peters J, Fleming S, Mullins JJ. Targeted inactivation of the Ren-2 gene in mice. Hypertension. 1996 Dec;28(6):1126-31.

Sigmund CD, Gross KW. Structure, expression, and regulation of the murine renin genes. Hypertension. 1991 Oct;18(4):446-57.
Simon G, Abraham G, Cserep G. Pressor and subpressor angiotensin II administration. Two experimental models of hypertension. Am J Hypertens. 1995 Jun;8(6):645-50.

Simon G, Illyes G, Csiky B. Structural vascular changes in hypertension: role of angiotensin II, dietary sodium supplementation, blood pressure, and time. Hypertension. 1998 Oct;32(4):654-60.

Siragy HM, Carey RM. The subtype-2 (AT2) angiotensin receptor regulates renal cyclic guanosine 3', 5'-monophosphate and AT1 receptor-mediated prostaglandin E2 production in conscious rats. J Clin Invest. 1996 Apr;97(8):1978-82.

Siragy HM, Carey RM. The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. J Clin Invest. 1997 Jul;100(2):264-9.

Skou JC, Esmann M. The Na,K-ATPase. J Bioenerg Biomembr. 1992 Jun;24(3):249-61.

Smith JD, Wong E, Ginsberg M. Cytochrome P450 1A1 promoter as a genetic switch for the regulatable and physiological expression of a plasma protein in transgenic mice. Proc Natl Acad Sci U S A. 1995 Dec;92(25):11926-30.

Snyder PM, Olson DR, Thomas BC. Serum and glucocorticoid-regulated kinase modulates Nedd4-2-mediated inhibition of the epithelial Na+ channel. J Biol Chem. 2002 Jan;277(1):5-8.

Song J, Hu X, Shi M, Knepper MA, Ecelbarger CA. Effects of dietary fat, NaCl, and fructose on renal sodium and water transporter abundances and systemic blood pressure. Am J Physiol Renal Physiol. 2004 Dec;287(6):F1204-12.

Soubrier F, Cambien F. The angiotensin I-converting enzyme gene polymorphism: implication in hypertension and myocardial infarction. Curr Opin Nephrol Hypertens. 1994 Jan;3(1):25-9.

Staahltoft D, Nielsen S, Janjua NR, Christensen S, Skøtt O, Marcussen N, Jonassen TE. Losartan treatment normalizes renal sodium and water handling in rats with mild congestive heart failure. Am J Physiol Renal Physiol. 2002 Feb;282(2):F307-15.

Steckelings U, Lebrun C, Qadri F, Veltmar A, Unger T. Role of brain angiotensin in cardiovascular regulation. J Cardiovasc Pharmacol. 1992;19 Suppl 6:S72-9.

Stoelting RK & Miller RD. Basics of ANESTHESIA. 5th ed. Philadelphia: Churchill Livingstone. 2007; 234.

Stokes JB, Sigmund RD. Regulation of αENaC mRNA by dietary NaCl and steroids: organ, tissue, and steroid heterogeneity. Am J Physiol. 1998 Jun;274(6 Pt 1):C1699-707.

Strautnieks SS, Thompson RJ, Gardiner RM, Chung E. A novel splice-site mutation in the gamma subunit of the epithelial sodium channel gene in three pseudohypoaldosteronism type 1 families. Nat Genet. 1996 Jun;13(2):248-50.

Suga SI, Phillips MI, Ray PE, Raleigh JA, Vio CP, Kim YG, Mazzali M, Gordon KL, Hughes J, Johnson RJ. Hypokalemia induces renal injury and alterations in vasoactive mediators that favor salt sensitivity. Am J Physiol Renal Physiol. 2001 Oct;281(4):F620-9.

Sun Y, Zhang J, Zhang JQ, Ramires FJ. Local angiotensin II and transforming growth factor-beta1 in renal fibrosis of rats. Hypertension. 2000 May;35(5):1078-84.

Swedberg K, Kjekshus J. Effects of enalapril on mortality in severe congestive heart failure: results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). Am J Cardiol. 1988 Jul;62(2):60A-66A.

Takahashi N, Chernavvsky DR, Gomez RA, Igarashi P, Gitelman HJ, Smithies O. Uncompensated polyuria in a mouse model of Bartter's syndrome. Proc Natl Acad Sci U S A. 2000 May;97(10):5434-9.

Takahashi N, Lopez ML, Cowhig JE Jr, Taylor MA, Hatada T, Riggs E, Lee G, Gomez RA, Kim HS, Smithies O. Ren1c homozygous null mice are hypotensive and polyuric, but heterozygotes are indistinguishable from wild-type. J Am Soc Nephrol. 2005 Jan;16(1):125-32.

Takenaka T, Harrison-Bernard LM, Inscho EW, Carmines PK, Navar LG. Autoregulation of afferent arteriolar blood flow in juxtamedullary nephrons. Am J Physiol. 1994 Nov;267(5 Pt 2):F879-87.

Talati G, Ohta A, Rai T, Sohara E, Naito S, Vandewalle A, Sasaki S, Uchida S. Effect of angiotensin II on the WNK-OSR1/SPAK-NCC phosphorylation cascade in cultured mpkDCT cells and in vivo mouse kidney. Biochem Biophys Res Commun. 2010 Mar;393(4):844-8.

Tang KM, Wang GR, Lu P, Karas RH, Aronovitz M, Heximer SP, Kaltenbronn KM, Blumer KJ, Siderovski DP, Zhu Y, Mendelsohn ME. Regulator of G-protein

signaling-2 mediates vascular smooth muscle relaxation and blood pressure. Nat Med. 2003 Dec;9(12):1506-12.

Tejera N, Gómez-Garre D, Lázaro A, Gallego-Delgado J, Alonso C, Blanco J, Ortiz A, Egido J. Persistent proteinuria up-regulates angiotensin II type 2 receptor and induces apoptosis in proximal tubular cells. Am J Pathol. 2004 May;164(5):1817-26.

Thekkumkara TJ, Cookson R, Linas SL. Angiotensin (AT1A) receptor-mediated increases in transcellular sodium transport in proximal tubule cells. Am J Physiol. 1998 May;274(5 Pt 2):F897-905.

Thomsen K, Schou M, Steiness I, Hansen HE. Lithium as an indicator of proximal sodium reabsorption. Pflugers Arch. 1969;308(2):180-4.

Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye JA, Smith RD. Angiotensin II receptors and angiotensin II receptor antagonists. Pharmacol Rev. 1993 Jun;45(2):205-51.

Titze J. Water-free sodium accumulation. Semin Dial. 2009 May-Jun;22(3):253-5.

Titze J, Lang R, Ilies C, Schwind KH, Kirsch KA, Dietsch P, Luft FC, Hilgers KF. Osmotically inactive skin Na+ storage in rats. Am J Physiol Renal Physiol. 2003 Dec;285(6):F1108-17.

Titze J, Machnik A. Sodium sensing in the interstitium and relationship to hypertension. Curr Opin Nephrol Hypertens. 2010 Jul;19(4):385-92.

Titze J, Shakibaei M, Schafflhuber M, Schulze-Tanzil G, Porst M, Schwind KH, Dietsch P, Hilgers KF. Glycosaminoglycan polymerization may enable osmotically inactive Na+ storage in the skin. Am J Physiol Heart Circ Physiol. 2004 Jul;287(1):H203-8.

Traynor T, Yang T, Huang YG, Krege JH, Briggs JP, Smithies O, Schnermann J. Tubuloglomerular feedback in ACE-deficient mice. Am J Physiol. 1999 May;276(5 Pt 2):F751-7.

Tsutsumi Y, Matsubara H, Ohkubo N, Mori Y, Nozawa Y, Murasawa S, Kijima K, Maruyama K, Masaki H, Moriguchi Y, Shibasaki Y, Kamihata H, Inada M, Iwasaka T. Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. Circ Res. 1998 Nov;83(10):1035-46.

Unger T, Badoer E, Ganten D, Lang RE, Rettig R. Brain angiotensin: pathways and pharmacology. Circulation. 1988 Jun;77(6 Pt 2):I40-54.
Urata H, Hoffmann S, Ganten D. Tissue angiotensin II system in the human heart. Eur Heart J. 1994 Dec;15 Suppl D:68-78.

Urata H, Nishimura H, Ganten D. Chymase-dependent angiotensin II forming systems in humans. Am J Hypertens. 1996 Mar;9(3):277-84.

Vallon V, Heyne N, Richter K, Khosla MC, Fechter K. [7-D-ALA]-angiotensin 1-7 blocks renal actions of angiotensin 1-7 in the anesthetized rat. J Cardiovasc Pharmacol. 1998 Jul;32(1):164-7.

Vallon V, Osswald H, Blantz RC, Thomson S. Potential role of luminal potassium in tubuloglomerular feedback. J Am Soc Nephrol. 1997 Dec;8(12):1831-7.

Vallon V, Schroth J, Lang F, Kuhl D, Uchida S. Expression and phosphorylation of the Na+-Cl- cotransporter NCC in vivo is regulated by dietary salt, potassium, and SGK1. Am J Physiol Renal Physiol. 2009 Sep;297(3):F704-12.

van der Mark J, Kline RL. Altered pressure natriuresis in chronic angiotensin II hypertension in rats. Am J Physiol. 1994 Mar;266(3 Pt 2):R739-48.

van Gilst WH, de Graeff PA, Wesseling H, de Langen CD. Reduction of reperfusion arrhythmias in the ischemic isolated rat heart by angiotensin converting enzyme inhibitors: a comparison of captopril, enalapril, and HOE 498. J Cardiovasc Pharmacol. 1986 Jul-Aug;8(4):722-8.

Van Vliet BN, Chafe LL, Antic V, Schnyder-Candrian S, Montani JP. Direct and indirect methods used to study arterial blood pressure. J Pharmacol Toxicol Methods. 2000 Sep-Oct;44(2):361-73.

Vanourková Z, Kramer HJ, Husková Z, Vanecková I, Opocenský M, Chábová VC, Tesar V, Skaroupková P, Thumová M, Dohnalová M, Mullins JJ, Cervenka L. AT1 receptor blockade is superior to conventional triple therapy in protecting against end-organ damage in Cyp1a1-Ren-2 transgenic rats with inducible hypertension. J Hypertens. 2006 Dec;24(12):2465-72.

Vasthare US, Irion GL, Carlsson C, Tuma RF. Differential effects of anesthetic agents on regional blood flow and central hemodynamic parameters in rats. Drug Development Research. 1988 March;14(1):59-65.

Vázquez E, Coronel I, Bautista R, Romo E, Villalón CM, Avila-Casado MC, Soto V, Escalante B. Angiotensin II-dependent induction of AT(2) receptor expression after renal ablation. Am J Physiol Renal Physiol. 2005 Jan;288(1):F207-13.

Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, Godbout K, Parsons T, Baronas E, Hsieh F, Acton S, Patane M, Nichols A, Tummino P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol Chem. 2002 Apr;277(17):14838-43.

Vitari AC, Thastrup J, Rafiqi FH, Deak M, Morrice NA, Karlsson HK, Alessi DR. Functional interactions of the SPAK/OSR1 kinases with their upstream activator WNK1 and downstream substrate NKCC1. Biochem J. 2006 Jul;397(1):223-31.
Von Thun AM, Vari RC, el-Dahr SS, Navar LG. Augmentation of intrarenal angiotensin II levels by chronic angiotensin II infusion. Am J Physiol. 1994 Jan;266(1 Pt 2):F120-8.

Wagner C, Kurtz A. Regulation of renal renin release. Curr Opin Nephrol Hypertens. 1998 Jul;7(4):437-41.

Wakabayashi S, Shigekawa M, Pouyssegur J. Molecular physiology of vertebrate Na+/H+ exchangers. Physiol Rev. 1997 Jan;77(1):51-74.

Walker LA, Buscemi-Bergin M, Gellai M. Renal hemodynamics in conscious rats: effects of anesthesia, surgery, and recovery. Am J Physiol. 1983 Jul;245(1):F67-74.

Wang CT, Chin SY, Navar LG. Impairment of pressure-natriuresis and renal autoregulation in ANG II-infused hypertensive rats. Am J Physiol Renal Physiol. 2000 Aug;279(2):F319-25.

Wang CT, Navar LG, Mitchell KD. Proximal tubular fluid angiotensin II levels in angiotensin II-induced hypertensive rats. J Hypertens. 2003 Feb;21(2):353-60.

Wang Q, Hummler E, Nussberger J, Clément S, Gabbiani G, Brunner HR, Burnier M. Blood pressure, cardiac, and renal responses to salt and deoxycorticosterone acetate in mice: role of Renin genes. J Am Soc Nephrol. 2002 Jun;13(6):1509-16.

Wang T, Giebisch G. Effects of angiotensin II on electrolyte transport in the early and late distal tubule in rat kidney. Am J Physiol. 1996 Jul;271(1 Pt 2):F143-9.

Wang T, Yang CL, Abbiati T, Schultheis PJ, Shull GE, Giebisch G, Aronson PS. Mechanism of proximal tubule bicarbonate absorption in NHE3 null mice. Am J Physiol. 1999 Aug;277(2 Pt 2):F298-302.

Wang Y, O'Malley BW Jr, Tsai SY, O'Malley BW. A regulatory system for use in gene transfer. Proc Natl Acad Sci U S A. 1994 Aug;91(17):8180-4.

Weinberger MH. Salt sensitivity of blood pressure in humans. Hypertension. 1996 Mar;27(3 Pt 2):481-90.

Weinberger MH, Fineberg NS. Sodium and volume sensitivity of blood pressure. Age and pressure change over time. Hypertension. 1991 Jul;18(1):67-71.

Weinberger MH, Fineberg NS, Fineberg SE, Weinberger M. Salt sensitivity, pulse pressure, and death in normal and hypertensive humans. Hypertension. 2001 Feb;37(2 Part 2):429-32.

Welches WR, Santos RA, Chappell MC, Brosnihan KB, Greene LJ, Ferrario CM. Evidence that prolyl endopeptidase participates in the processing of brain angiotensin. J Hypertens. 1991 Jul;9(7):631-8.

Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007 Jun;447(7145):661-78.

Wharton J, Morgan K, Rutherford RA, Catravas JD, Chester A, Whitehead BF, De Leval MR, Yacoub MH, Polak JM. Differential distribution of angiotensin AT2 receptors in the normal and failing human heart. J Pharmacol Exp Ther. 1998 Jan;284(1):323-36.

Whelton PK. Epidemiology of hypertension. Lancet. 1994 Jul;344(8915):101-6.

Whitworth CE, Fleming S, Cumming AD, Morton JJ, Burns NJ, Williams BC, Mullins JJ. Spontaneous development of malignant phase hypertension in transgenic Ren-2 rats. Kidney Int. 1994 Dec;46(6):1528-32.

Whitworth CE, Fleming S, Kotelevtsev Y, Manson L, Brooker GA, Cumming AD, Mullins JJ. A genetic model of malignant phase hypertension in rats. Kidney Int. 1995 Feb;47(2):529-35.

Whitworth JA; World Health Organization, International Society of Hypertension Writing Group. 2003 World Health Organization (WHO)/International Society of Hypertension (ISH) statement on management of hypertension. J Hypertens. 2003 Nov;21(11):1983-92.

Williams B, Poulter NR, Brown MJ, Davis M, McInnes GT, Potter JF, Sever PS, Thom SM; BHS guidelines working party, for the British Hypertension Society. British Hypertension Society guidelines for hypertension management 2004 (BHS-IV): summary. BMJ. 2004 Mar 13;328(7440):634-40. Erratum in: BMJ. 2004 Apr;328(7445):926.

Williams DE, Prieto MC, Mullins JJ, Navar LG, Mitchell KD. AT1 receptor blockade prevents the increase in blood pressure and the augmentation of intrarenal ANG II levels in hypertensive Cyp1a1-Ren2 transgenic rats fed with a high-salt diet. Am J Med Sci. 2010 Apr;339(4):356-61.

Wilson FH, Disse-Nicodème S, Choate KA, Ishikawa K, Nelson-Williams C, Desitter I, Gunel M, Milford DV, Lipkin GW, Achard JM, Feely MP, Dussol B, Berland Y, Unwin RJ, Mayan H, Simon DB, Farfel Z, Jeunemaitre X, Lifton RP. Human hypertension caused by mutations in WNK kinases. Science. 2001 Aug;293(5532):1107-12.

Wolf-Maier K, Cooper RS, Banegas JR, Giampaoli S, Hense HW, Joffres M, Kastarinen M, Poulter N, Primatesta P, Rodríguez-Artalejo F, Stegmayr B, Thamm M, Tuomilehto J, Vanuzzo D, Vescio F. Hypertension prevalence and blood pressure

levels in 6 European countries, Canada, and the United States. JAMA. 2003 May;289(18):2363-9.

Woolfson RG, de Wardener HE. Primary renal abnormalities in hereditary hypertension. Kidney Int. 1996 Sep;50(3):717-31.

Yamamoto K, Chappell MC, Brosnihan KB, Ferrario CM. In vivo metabolism of angiotensin I by neutral endopeptidase (EC 3.4.24.11) in spontaneously hypertensive rats. Hypertension. 1992 Jun;19(6 Pt 2):692-6.

Yang CL, Zhu X, Wang Z, Subramanya AR, Ellison DH. Mechanisms of WNK1 and WNK4 interaction in the regulation of thiazide-sensitive NaCl cotransport. J Clin Invest. 2005 May;115(5):1379-87.

Yang SS, Morimoto T, Rai T, Chiga M, Sohara E, Ohno M, Uchida K, Lin SH, Moriguchi T, Shibuya H, Kondo Y, Sasaki S, Uchida S. Molecular pathogenesis of pseudohypoaldosteronism type II: generation and analysis of a Wnk4(D561A/+) knockin mouse model. Cell Metab. 2007 May;5(5):331-44.

Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgin J, Shaul PW, Thoren P, Smithies O, Gustafsson JA, Mendelsohn ME. Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. Science. 2002 Jan;295(5554):505-8.

Zou AP, Wu F, Cowley AW Jr. Protective effect of angiotensin II-induced increase in nitric oxide in the renal medullary circulation. Hypertension. 1998 Jan;31(1 Pt 2):271-6.

Zou LX, Imig JD, Hymel A, Navar LG. Renal uptake of circulating angiotensin II in Val5-angiotensin II infused rats is mediated by AT1 receptor. Am J Hypertens. 1998 May;11(5):570-8.

Zou LX, Imig JD, von Thun AM, Hymel A, Ono H, Navar LG. Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. Hypertension. 1996 Oct;28(4):669-77.